TAMPERE UNIVERSITY OF TECHNOLOGY

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MICROPARTICLES IN DIFFERENTIATION OF RETINAL PIGMENT EPITHELIAL CELLS FROM HUMAN PLURIPOTENT STEM CELLS

Master of Science Thesis

Examiners: Professor Minna Kellomäki,
PhD Tanja Ilmarinen
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ABSTRACT

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The number of people affected by degenerative diseases of the retina increases notably due to the longer life expectancy. The weakening of the sight concerns especially older people, who suffer from degeneration of the retina and its retinal pigment epithelium (RPE) monolayer. Currently, these degenerative diseases of the retina are incurable. Cell transplantation of healthy RPE cells is considered to be a potential treatment for these diseases. Due to the unlimited self-renewal capacity and pluripotency human pluripotent stem cells (hPSCs) are considered as possible cell sources for transplantation purposes. However, the current differentiation protocols for RPE cells from hPSCs are inefficient and result in low yields.

The aim of this thesis was to study whether biodegradable poly(L-lactide) (PLLA) microparticles and commercial biostable cellulose anion exchange diethylaminoethyl (DE) 53 microparticles could be exploited in increasing the efficacy of differentiation of hPSCs into RPE cells. In addition, the potential of the microparticle culture system for stem cell maintenance in feeder-free conditions was evaluated. The attachment and proliferation of hPSCs on microparticles were analyzed by immunofluorescence analysis and microscopy. The success of differentiation of RPE from stem cells in microparticle cultures was evaluated by inspecting the appearance of pigmentation in cultures as well as by investigating the expression of RPE maturation markers on gene expression levels by RT-PCR.

The PLLA microparticles did not support the attachment and proliferation of hPSCs in feeder-free conditions. The poor performance of PLLA particles in cell culture studies might be due to the hydrophobic nature of the particle surfaces. In contrast, hPSCs attached, proliferated and maintained their pluripotency on DE 53 particles. Furthermore, the hPSCs were successfully differentiated into RPE cells on these DE 53 particles. However, the differentiation efficacy did not outperform the conventional differentiation protocol.

This was the first study in which the hPSCs were successfully differentiated into RPE cells on microparticles. Microparticles are far from being routinely used in hPSC maintenance and differentiation to mature cell types. More long-term and large-scale studies need to be conducted in the future. Nevertheless, at the moment they seem appealing and promising with a possibility to grow stem cells in large-scale and cost effectively. The microparticle culture systems set challenges for the cell analysis due to their 3-dimensional structure and autofluorescence property. Therefore, novel methods for characterization, analysis and imaging of microparticle culture systems should be developed.

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Väestön ikääntyessä myös rappeumasairauksien esiintyvyys lisääntyy. Verkkokalvon ikärappeumasta kärsivät yleensä ikääntyneet yli 60-vuotiaat ihmiset. Verkkokalvon ikärappeumassa verkkokalvon pigmenttiepiteeli (RPE) on vaurioitunut. Tällä hetkellä ikärappeumaan ei ole parannuskeinoa, ja olemassa olevat hoidot vain hidastavat sairauden etenemistä. Terveiden RPE-solujen siirtoa vaurioituneelle alueelle pidetään lupaavana hoitokeinona verkkokalvon ikärappeumaan. Ihmisen erittäin monikykyiset kantasolut (hPSCs) ovat jatkuvasti jakaantuvia soluja, joilla on kyky erilaistua useiksi solutyypeiksi. Kuitenkin näiden solujen erilaistaminen RPE-soluiksi nykyisillä menetelmillä on tehotonta, ja erilaistuksen saannot ovat pieniä.

Tämän diplomityön tavoitteena oli tutkia voidaanko biohajoavia poly(L-laktidi) (PLLA) ja kaupallisia hajoamattomia DE 53 mikropartikkeleita hyödyntää RPE-solujen erilaistuksen tehostamisessa kantasoluista. Myös mikropartikkeleiden soveltuvuus kantasolujen ylläpitämiseen ilman ihmisen esinahan fibroblasteja tukisoluina oli tutkimuksen kohteena. Kantasolujen kiinnittymistä ja lisääntymistä mikropartikkeleille analysoitiin immunofluoresenssitekniikalla ja mikroskopoimalla. Solujen erilaistumista mikropartikkeleilla seurattiin tarkkailemalla pigmentoituneiden solujen ilmestymistä viljelmiin, sekä kypsien RPE-solujen markkereiden ilmaantumista geenitasolla RT-PCR:llä.

Tutkimuksessa selvisi, että biohajoavat PLLA-mikropartikkelit eivät tue hPSC-solujen kiinnittymistä ja lisääntymistä. Tämä johtuu todennäköisesti PLLA-partikkeleiden hydrofobisesta pinnasta. Sitä vastoin hPSC-solut kiinnittyivät, lisääntyivät ja pysyivät monikykyisinä kaupallisilla DE 53-partikkeleilla. Lisäksi DE 53-partikkelit tukivat hPSC-solujen erilaistumista RPE-soluiksi erilaistuksen tehostustutkimuksessa. Erilaistus mikropartikkeliviljelmässä ei ollut kuitenkaan tehokkaampaa kuin perinteisessä käytössä olevassa erilaistusmenetelmässä.

Tämä tutkimus oli tietämämme ensimmäinen, jossa hPSC-soluja erilaistettiin onnistuneesti RPE-soluiksi mikropartikkeliviljelmässä. Perusteellisempia pitkän aikavälin tutkimuksia mikropartikkeleiden soveltuvuudesta kantasolujen viljelyyn ja erilaistukseen tulisi tehdä jatkossa. Kantasolujen kasvatus mikropartikkeliviljelmissä mahdollistaisi toimiessaan näiden solujen laajamittaisen tuotannon kustannustehokkaasti. Tällä hetkellä suuri haaste tutkimuksessa on solujen analysointi mikropartikkeleiden vahvan autofluoresenssin ja kolmiulotteisen rakenteen vuoksi.

PREFACE

This Master of Science thesis was carried out in the Ophthalmology group of the Institute of Biosciences and Medical Technology (BioMediTech) at the University of Tampere.

First, I would like to thank Associate Professor Heli Skottman, the group leader of Ophthalmology, for the opportunity to conduct my thesis in the fascinating field of regenerative medicine and ophthalmology. Second, I would like to express my graditute to our collaborator Professor Joachim Loo from Nanyang Technological University for providing the microparticle preparation protocol and polymers for this study. I would also like to thank Professor Minna Kellomäki for the advice regarding biomaterials.

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Tampere, December 2011

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ABBREVIATIONS

ACT	Advanced Cell Technology
AMD	Age-related macular degeneration
ASC	Adult stem cell
BAL	Bioartificial liver
BDNF	Brain-derived growth factor
BEST	A gene expressin bestrophin
bFGF	Basic fibroblast growth factor
BM	Bruch's membrane
BMP	Bone morphogenetic protein
BRB	Blood-retinal barrier
BSA	Bovine serum albumin
cDNA	Complementary deoxyribonucleic acid
CM	Conditioned media
c-Myc	A transcription factor
CNV	Choroid neovascularisation
CRALBP	Cellular retinaldehyde-binding protein
DAPI	4',6-diamino-2-phenylindole
DCM	Dicholoromethane
DE	Diethylaminoethyl
DM-	Differentiation medium
DNA	Deoxyribonucleic acid
DPBS	Dulbecco's Phosphate Buffered Saline
EB	Embryoid body
ECM	Extra cellular matrix
EGF	Epidermal growth factor
EL	Elastin layer
FGF	Fibroblast growth factor
FBS	Fetal bovine serum
FOXA2	Forkhead box protein A2
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GDNF	Glial cell-derived neurotrophic factor
hFF	Human foreskin fibroblast
hEB	Human embryoid body
ESC	Embryonic stem cell
hESC	Human embryonic stem cell
HH	Hedgehog
hiPSC	Human induced pluripotent stem cell
hPSC	Human pluripotent stem cell. Comprises embryonic and induced pluripotent stem cells.
ICL	Inner collagenous layer

ICM	Inner cell mass
IGF-I	Insulin-like growth factor-I
I.V.	Inherent viscosity
IVF	*In vitro* fertilization
Klf-4	Krueppel-like factor 4
LIF	Leukemia inhibitory factor
Lin28	A marker of undifferentiated human embryonic stem cells
MEF	Mouse embryonic fibroblast
MITF	Microphthalmia-associated transcription factor
MSC	Mesenchymal stem cell
mESC	Mouse embryonic stem cell
Nanog	A transcription factor and a marker of undifferentiated cells.
NaOH	Sodium hydroxide
NGF	Nerve growth factor
NSC	Neural stem cell
NT-3	Neurotrophin-3
OCL	Outer collagenous layer
OCT4	Octamer-4
Otx 2	Orthodenticle homeobox 2
PAM	Pharmacologically active microparticle
PA 6	Mesenchymal bone marrow cells
PCL	Poly(ε-caprolactone)
PDGF	Platelet derived growth factor
PDMS	Poly(dimethylsiloxane)
PDT	Photodynamic therapy
PEDF	Pigment epithelium derived factor
PEG	Polyethylene glycol
PEI	Polyethyleneimine
PFA	Paraformaldehyde
PGA	Polyglycolide
PLA	Polylactide
PLGA	Polylactide-co-glycolide
PLLA	Poly(L-lactide)
PVA	Polyvinylalcohol
RA	Retinoic acid
ROCK	p160-Rho-associated coiled kinase
RP	Retinis pigmentosa
RPE	Retinal pigment epithelium
RPE 65	Retinal pigment epithelium-specific 65 kDa protein
RT	Room temperature
RT-PCR	Reverse transcriptase polymerase chain reaction
SDIA	Stromal cell-derived inducing activity

SEM	Scanning electron microscopy
SOX2	Sex determining region Y- box 2
SOX17	Sex-determining region Y- box 17
SR	Serum replacement
SSE-3	State-specific embryonic antigen-3
SSE-4	State-specific embryonic antigen-4
TCEP	Reverse transcriptase polymerase chain reaction
TGF-β	Transforming growth factor beta
VEGF	Vascular endothelial growth factor
VSX2	Visual system homeobox 2

1. INTRODUCTION

Vision is a prerequisite for a good life quality and independency. Today, there are approximately 180 million visually impaired persons in the world. Approximately 45 million of these are completely blind, and the rest are more or less weak sighted. The weakening of the sight concerns especially older people, who suffer from degeneration of the retina and its retinal pigment epithelium (RPE) monolayer. As the population is getting older due to the higher life expectancy, the number of people dealing with degenerative diseases of the retina, increases notably. (Finnish Federation of the Visually Impaired 2011)

In many cases of retinal degeneration, the inner layers of the retina, associated neural and glial cells and the optic nerve maintain their functionality and organized structure for a period of time. Therefore, by introducing a population of healthy cells, with the capacity to promote photoreceptor survival, into the damaged subretinal area, the vision could be preserved. For instance, age-related macular degeneration (AMD) is the most common cause of blindness among people over 60 years of age. Currently, there is neither prophylactic treatment nor cure for this degenerative disease. Transplantation of healthy RPE cells is considered as a very potential future therapy method for retinal degenerative diseases such as AMD. (Gehrs et al. 2006; Hynes et al. 2010)

The effectiveness of the cell transplantation to treat degenerative retinal diseases has been already proven with autologous cells. However, there are not usually enough healthy cells left in degenerative retinal disease patients for transplantation purposes. (Binder et al. 2002) Therefore, human pluripotent stem cells (embryonic and induced stem cells, hPSCs) are considered to be potential cell sources for transplantation purposes. Nevertheless, traditional culture methods of hPSCs are impractical or unable to produce necessary scale-up of the cells for transplantation and research purposes. (Martin et al. 2011; Rowland et al. 2011) The currently used protocols for RPE cell differentiation from stem cells are based on spontaneous differentiation and result in low yields (Klimayanska et al. 2004; Rowland et al. 2011). Therefore, the search for more effective culture systems and differentiation protocols arouse huge interest among the field of ophthalmology and tissue engineering.

Diameter of microparticles is in microscale. These small particles have been used for cell culture of anchorage dependent cells for decades with great success (van Wezel 1967; Martin et al. 2011). Microparticles offer great advantages over conventional 2-dimensional (2D) culture systems such as a larger surface area. In addition, they overcome the need for large quantities of culture media, space expense, inefficient gas-liquid oxygen transfer, presence of concentration gradients and

difficulties in monitoring and control. The utility of microparticles in cell culture has been shown with mature cells, but modifying microparticle culture system for hESCs is more difficult. (Martin et al. 2011; Sun et al. 2011)

The aim of this thesis was to study if self-prepared biodegradable microparticles and commercial biostabile microparticles could be used for increasing the efficacy of differentiation of hPSCs into RPE cells. In addition, the potential of the microparticle culture system for stem cell maintenance in feeder-free conditions was evaluated. In this study, biodegradable poly(lactide-co-glycolide) (PLGA) and poly(L-lactide) (PLLA) microparticles were prepared by a single emulsion-based method. The characteristics and performance of these self-prepared particles in cell culture and differentiation experiments were compared to commercial cellulose anion exchange diethylaminoethyl (DE) 53 microparticles.

This thesis consists of a theoretical and an experimental part. In the theoretical part, background information about the structure and functions of RPE and retinal degenerative diseases are introduced. In addition, the potential of hPSCs as a cell source for tissue engineering applications, and the differentiation protocols for RPE from stem cells are illustrated. Furthermore, the concept of microparticles, their preparation methods and current use in cell culture and tissue engineering are presented. In the experimental part, the course of the study from particle preparation to differentiation of hPSCs on microparticles is reported in detail. Finally, the results of these experiments are reported, discussed and concluded.

THEORETICAL PART

2. RETINA

The sight is vital for our understanding of the surrounding environment. In fact, the eye contains approximately 70% of the sensory cells of the body. The eye is a complex structure which consists of epithelial, mesenchymal, connective and neural tissue. It is a highly specialized organ capable of photoreception, a process which involves the conversion of light energy into nerve action potentials. (Haug et al. 1999; Young et al. 2006) In next chapters, the structure of the eye and the retina are introduced with special focus on the architecture and function of the retinal pigment epithelium in the back of the eye.

2.1. The structure of the eye and the retina

The diameter of the eyeball in humans is approximately 2.5 cm. The outer layer of the eyeball is a strong fibroelastic capsule, sclera. However, in front of the eye the sclera transforms into a transparent cornea. The cornea is mostly responsible for the refraction of the light in the eye, and it roughly focuses the light onto the retina. Inside the sclera, there is a loose vascular tissue layer called choroid. Interestingly, the choroid is heavily pigmented, which absorbs the light passing through the retina. The choroid merges with ciliary body in front of the eye. The iris is also an extension of the choroid and it is also heavily pigmented. The color of the eyes changes between individuals according to the amount of pigment in the iris. An aperture of the eye is called a pupil. Behind the pupil there is a clear and elastic lens, which attaches to the ciliary body with suspensory ligaments. The lens enables the accurate refraction of the light into the retina by changing its form. Furthermore, the lens divides the eyeball into smaller anterior chamber and gel-like vitreous body. The light that enters the eye is focused on the retina, which forms the inner lining of the posterior compartment of the eye. The optic nerve consists of the afferent nerve fibres from the retina, and it leaves the eye through a part of the sclera. (Haug et al. 1999; Young et al. 2006) The structure of the eye is presented in the Figure 1.

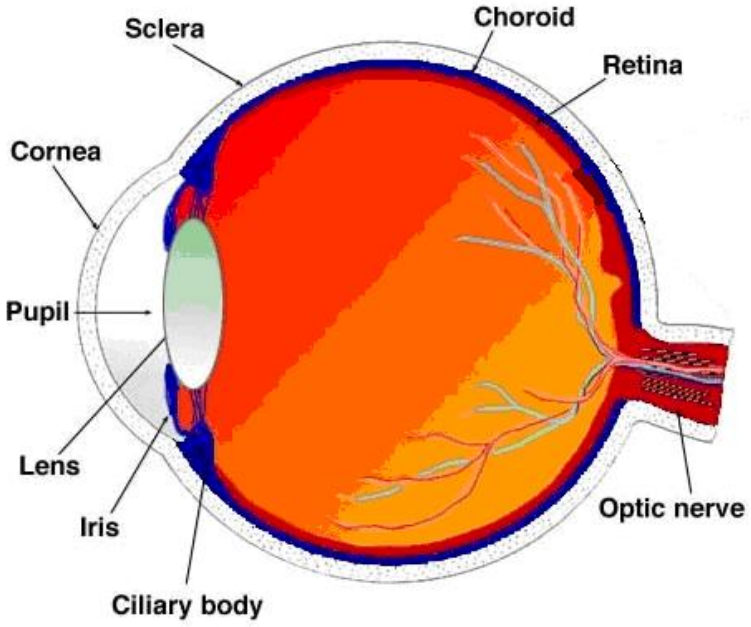

Figure 1. *The structure of the eye. (Modified from the source Kolb et al. 2005)*

The light is absorbed in retina, which contains three types of cells: neurones, pigmented epithelial cells and supporting cells. The retina is approximately 0.5 mm thick and lines the back of the eye. All vertebrae retinas are composed of three layers of nerve cell bodies and two layers of synapses. The outermost layer of the retina consists of the RPE cells, which lay on top of the Bruch's membrane (BM) and separates the retina from the choroid. The rod and cone photoreceptors embed their tips into the microvilli of RPE cells forming a photoreceptor layer. The photoreceptor layer includes a thin eosinophilic structure known as the outer limiting membrane, which separates the rod and cone processes of the photoreceptors from the densely packed nuclei called outer nuclear layer. The outer plexiform layer contains synaptic connections between photoreceptor cells and integrating neurones, whereas the cell bodies of these neurones, such as bipolar, horizontal, and amacrine cells, lie in the inner nuclear layer. Besides these integrating neurones, Müller glia cells, which are analogous to the neuroglia in the central nervous sytem, embrace the retinal neurones filling all the intervening spaces. The integrating neurones make synaptic connections with dendrites of ganglion cells in the inner plexiform layer. The axons of retinal ganglion cells form the optic tract. Finally, the inner limiting membrane distinguishes the retina from the vitreous body. (Young et al. 2006; Kolb et al. 2007) A simplified picture of the structure of the retina is illustrated in the Figure 2.

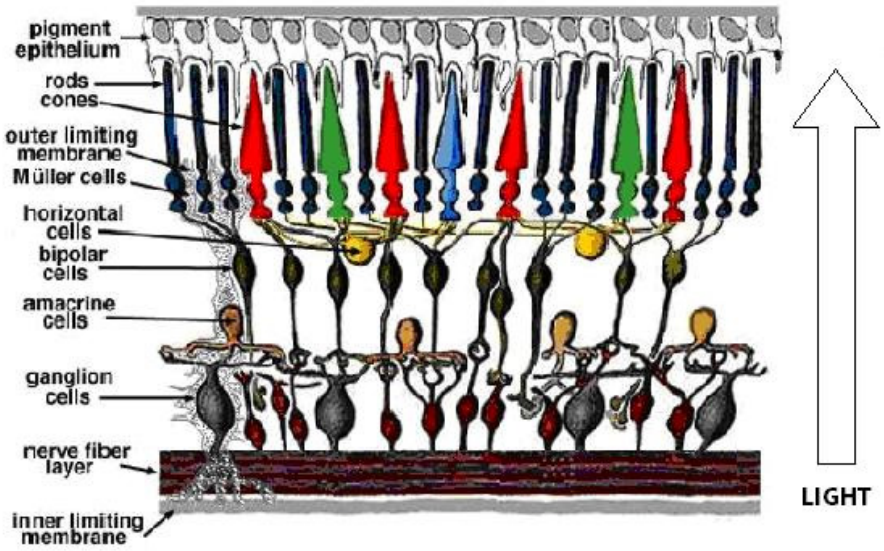

Figure 2. *A simplified picture of the structure of retina. (Modified from the source Kolb et al. 2005)*

2.2. The retinal pigment epithelium

RPE is a monolayer of darkly pigmented cells which are anatomically situated between the neural retina and the choriocapillaris. RPE arises from the neuroectoderm and it is considered to be a part of the retina. It plays a crucial role in support and function of retina and its photoreceptors, such as formation of the blood-retinal barrier (BRB), regeneration of visual pigment, transportation of nutrients, and absorption of stray light. Anatomically the RPE is aligned so that the apical membrane of RPE is face to the photoreceptors whereas basal surfaces of the cells rest on BM. (Simó et al. 2010)

2.2.1. Development of RPE in vertebrates

Organogenesis of the eye is a multiphase process that begins with the formation of optic vesicles after which the distal domain of the vesicles folds inward in the development of the bilayered optic cup. The formation of the optic vesicles and the optic cup takes place in the ventral forebrain during the final stages of neural tube formation. This process is a delicate and well-coordinated process between neuroepithelium, surface ectoderm and extraocular mesenchyme. The neural retina develops from inner layer of the optic cup whereas the RPE is derived from the outer layer. Tissue-tissue interactions, which are mediated by extrinsic and intrinsic factors and signals such as transcription factors, play a crucial role in the differentiation of ocular tissues starting from the optic vesicle stage. (Bharti et al. 2006; Fuhrmann 2010) The formation of the optic cup in early eye development is illustrated in Figure 3.

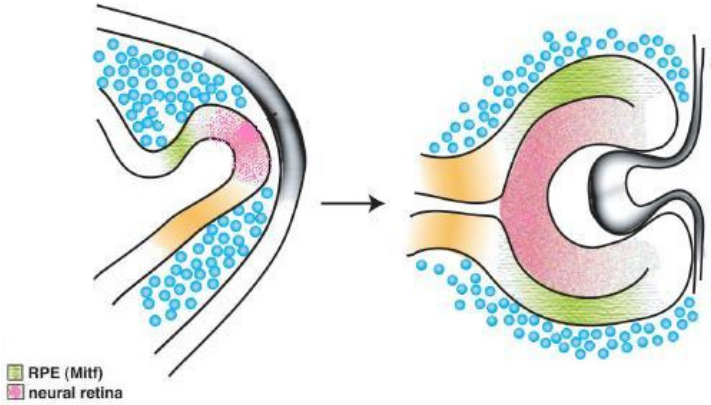

Figure 3. *Formation of the optic cup and RPE in early eye development. (Modified from the source Fuhrmann 2010)*

The RPE is specified at the early optic vesicle stage already before the pigmentation comes visible. The early markers on gene expression level that show domain-specific expression for retina and RPE are the visual system homeobox 2 (VSX2) and microphthalmia-associated transcription factor (MITF). (Fuhrmann 2010) During human RPE development, the first signs of pigmentation are seen in the external layer of the optic cup approximately on post-ovulatory weeks 5 to 6. (Vugler et al. 2007) Transcription factors MITF and orthodenticle homeobox 2 (OTX2) play crucial roles in RPE specification; *MITF* is the first gene that is specifically expressed in the developing RPE in the optic vesicle and it is also a key regulator of pigment cell development in the RPE, whereas OTX2 activates expression of pigment genes in cooperation with MITF. (Bharti et al. 2006; Fuhrmann 2010) Transcription factors and functional proteins involved in development of the RPE and photoreceptors are listed in Figure 4.

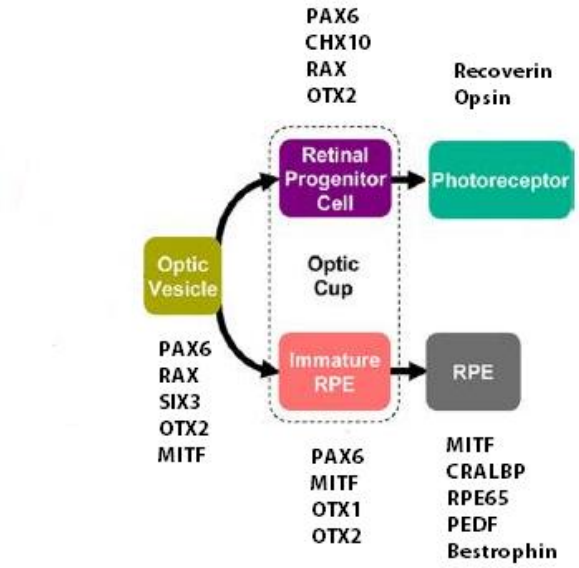

Figure 4. *Expressions of transcription factors and functional proteins in development of the eye. (Modified from the sources Meyer et al. 2009 & Vaajasaari et al. 2011)*

The most important extrinsic factors in RPE development are proteins of the transforming growth factor β (TGFβ) superfamily, such as bone morphogenetic proteins (BMPs); factors of the hedgehog (HH) family; and fibroblast growth factors (FGFs). For instance, the surface ectoderm expresses BMPs which have shown to induce MITF expression and induce RPE fate in the optic vesicle. (Bharti et al. 2006; Müller et al. 2007) Instead, basic fibroblast growth factor (bFGF) promotes the developing RPE to transdifferentiate into neural progenitor cells. In addition, the Wnt signalling pathway affects on the undifferentiated retinal progenitor by inducing the formation of the laminar structure of the retina. (Vugler et al. 2007)

2.2.2. The RPE cells

The RPE cells possess the anatomic features of a typical monolayer epithelium with tightly packed cubical or columnar cells and microvilli on the apical side of the cells. The apical microvilli surround the light-sensitive outer segments of the photoreceptors. (Strauss 2005) The cytoplasm of the cells is compartmentalized along an apico-basal axis; a well-developed epithelium has cell organelles and cytoskeletal molecules localized to specific sub-cellular positions. (Klimanskaya et al. 2004; Burke et al. 2008) Furthermore, the polarized RPE cells have a substantially regular hexagonal organization when viewed from above the surface. (Burke 2008) Mature RPE cells do not normally divide, but they can start to proliferate in response to injury or photocoagulation. In these cases, the RPE cells nearby the damaged area flatten and proliferate forming a continuous monolayer. (Klimanskaya et al. 2004) The cobblestone-like morphology of RPE cells and RPE monolayer are illustrated in Figure 5.

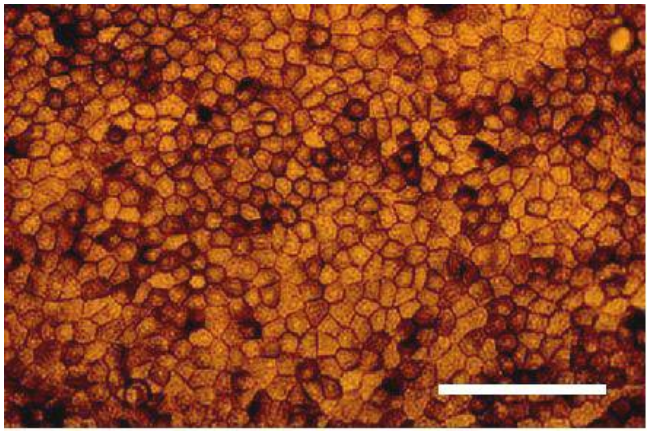

Figure 5. *The cobblestone-like structure and pigmentation of human foetal RPE monolayer. Scale bar 100 µm. (Modified from the source Volobоueva et al. 2005)*

Several studies have shown that RPE can form fibroblast-like cells that can later revert to their original RPE cell morphology. (Lee et al. 2001) One of the most obvious markers of mature RPE cells is the formation of brown pigment called melanin inside the cells. The pigment is inside small granules, melanosomes. When RPE cells are

propagated in the culture, they lose their pigmentation as melanosomes are exocytosed or diluted by cell division. However, RPE cells are able to gain the lost pigmentation in a process called re-melanization. Nevertheless, biosynthesis of melanin is a slow process which requires weeks of confluency of the RPE cells. (Burke et al. 2008; Gamm et al. 2008) In between the RPE cells there are tight, adherens and gap junctions, which create a barrier between the subretinal space and choriocapillaris. For RPE the paracellular resistance is 10 times higher than the transcellular recistance. (Strauss 2005)

2.2.3. The functions of RPE

Sense of sight is a delicate and complicated system in which the RPE has a significant and vital role. First and foremost RPE participates in light absorption and protection against photo-oxidation. The RPE increases optical quality by forming a heavily dark pigmented lining of the inner bulbus, which contributes to absorption of scattered light. In the eye, light enters via pupil and is focused onto the macula lutea by the lens. Therefore the light energy is concentrated onto the retina. The RPE contains a complex composition of several pigments that absorb light in specialized wavelengths, such and melanin and lipofuscin. The general light absorption occurs via melanin in melanosomes. In addition, this light absorption is supplemented by pigments in the photoreceptors, such as lutein and zeaxanthin. These pigments absorb blue light, which appears to be most dangerous for the RPE cells. Melanosomes and blue light absorpting pigments are only responsible for the absorption of approximately 60 per cent of light energy (Strauss 2005; Simó et al. 2010) Besides light absorption, the RPE protects the retina by counterbalancing the high oxidative stress. This is mediated by the high amounts of antioxidants, such as superoxide dismutase and catalase in RPE. (Miceli et al. 1994)

The RPE has a vital role in the visual cycle. Light transduction is initiated by the absorbtion of light by rhodopsin. Rhodopsin is composed of opsin and a chromophore 11-*cis*-retinal. When light is absorbed, the conformation of 11-cis-retinal changes into all-*trans*retinal. All-*trans*retinal is metabolized into all-*trans*retinol by photoreceptors and transported to the RPE. In the RPE, the all-*trans*retinol is reisomerized to 11-*cis*-retinal and then delivered back to the photoreceptors. The protein retinal pigment epithelium specific protein 65 kDa (RPE65) is responsible for the isomerization of the all-*trans*-retinaldehyde to its photoactive 11-*cis*-retinaldehyde. In addition, a cellular retinaldehyde binding protein (CRALBP) is related to the retinoid metabolism. Both proteins are molecular markers for RPE. (Klimanskaya et al. 2004; Burke et al. 2008; Simó et al. 2010) The functions of RPE are illustrated in Figure 6.

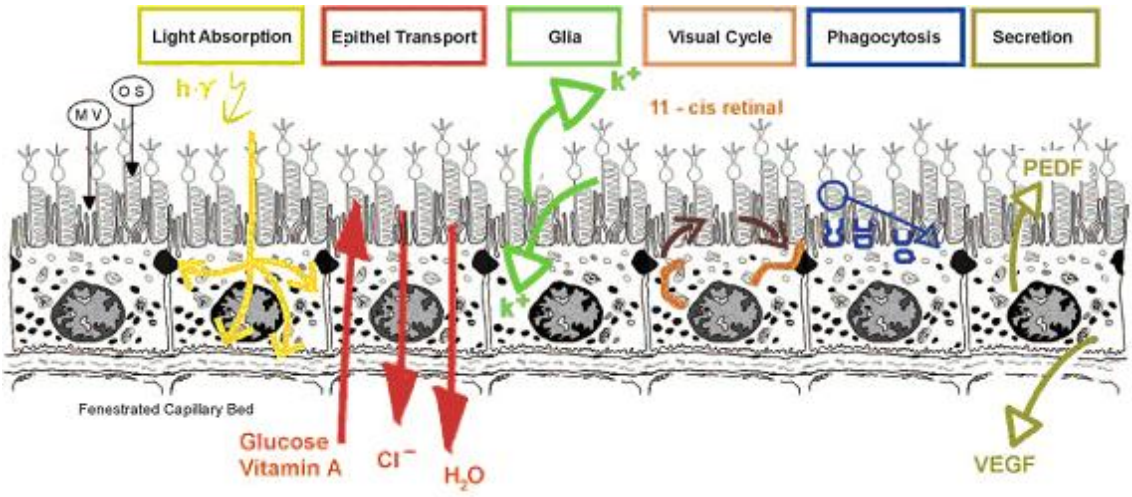

Figure 6. *The functions of the RPE. (Strauss 2005)*

The RPE transports nutrients and ions between the choriocapillaris and photoreceptors. This epithelial transport through RPE is therefore bidirectional. Energy for transepithelial transport is provided by Na^+- K^+-ATPase on the apical membrane of RPE cells. Neurons and photoreceptors in retina produce large amounts of water as a result of their metabolic turnover. In addition, intraocular pressure moves water from the vitreous body into the retina. Thus, there is a constant need for removal of water from retina by RPE cells. The transport of water is mainly driven by a transport of Cl^- and K^+. (Hamann 2002) A protein that plays an important role in chloride conductance and calcium signalling in the RPE cells is bestrophin. Bestrophin is also a commonly used molecular marker for RPE cells. (Klimanskaya et al. 2004; Burke et al. 2008) Due to the high paracellular resistance of RPE epithelium, water transport occurs mainly transcellularly and it is facilitated by aquaporins. (Hamann et al. 1998) Furthermore, metabolic end products from the photoreceptors, such as lactid acid, are eliminated by the RPE into the blood circulation. (Strauss 2005) RPE is also responsible for the transportation of nutrients, such as glucose, retinol and fatty acids from blood into the retina. The import of retinol is extremely important to ensure enough retinol to the photoreceptors. The vitamin A uptake from the blood constitutes additional supply to the visual and retinoid cycle. (Baehr et al. 2003)

Another important function of the RPE is the phagocytosis of shed photoreceptor outer segments. Exposure of photoreceptors to intense levels of light leads to accumulation of photodamaged proteins and lipids. Thus, to preserve the excitability of photoreceptors, their outer segments undergo constant renewal. These damaged tips of photoreceptor outer segments are phagocytosed by the RPE in such manner that essential molecules, such as retinol, are redelivered to photoreceptors. The RPE is also known to produce and secrete growth factors as well as essential factors for the maintenance of the retina and choriocapillaris. For instance, the RPE is able to secrete pigment epithelium derived factor (PEDF), vascular endothelial growth factor (VEGF), FGFs, TGF-β, insulin-like growth factor-I (IGF-I), nerve growth factor (NGF),

brain-derived growth factor (BDNF), neurotrophin-3 (NT-3), platelet-derived growth factor (PDGF) and chemokins. Among these numerous factors, PEDF and VEGF seem to be the most significant ones. For instance, PEDF has a property of inhibiting angiogenesis. (Strauss 2005, Simó et al. 2010)

2.2.4. Bruch's membrane

Bruch's membrane (BM) is an extraordinary pentalaminar membrane composed of extracellular matrix proteins between the RPE and the choroid. Due to its unique location and structure, BM plays a vital role in cell-cell communication, cellular differentiation, proliferation and migration, tissue remodelling as well as in shaping of pathological processes. The main components of BM are laminin, collagen type IV, fibronectin and collagen I. BM is an acellular layer, and most probably its extracellular matrix (ECM) constituent production is dependent on the adjacent RPE and choroidal cells. (Booij et al. 2010)

The three most important functions of the BM consist of regulating the diffusion of molecules between the choroid and the RPE, providing physical support for the anchorage dependent RPE cell adhesion, migration and differentiation as well as acting as a division barrier restricting choroidal and retinal cellular migration. The BM acts as a semi-permeable barrier for reciprocal exchange of biomolecules between retina and choroid. This diffusion of molecules is mainly passive due to the acellular nature of BM. Several factors affect the diffusion over BM, such as age and location in retina. Furthermore, diffusion through the BM depends also on hydrostatic pressure on both sides of the BM and concentration of specific biomolecules and inorganic ions. Biomolecules such as nutrients, lipids, pigment precursors, vitamins, oxygen, minerals, anti-oxidant components and serum constituents are taken up by the RPE via BM from the bloodstream. In contrast, CO_2, water, ions, oxidized lipids, oxidised cholesterol and other waste products from the RPE pass the BM to choroid and bloodstream. The role of BM as substrata for the RPE is vital. The adhesion of RPE cells on BM is mediated by integrin cell surface receptors. These integrin receptors are a group of RPE basal membrane proteins that are able to bind a number of extracellular components such as laminin and type IV collagen. (Booij et al. 2010)

2.3. Retinal disorders

As the population is ageing and the average life expectancy is rising over 80 years in the developed countries, the amount of people suffering from degenerative diseases is increasing. 90% of the visually impaired persons live in the developing countries. Degenerative diseases affecting the retina eventually lead to irreversible weakening of vision and may cause blindness. Today, there are approximately 180 million visually impaired persons in the world out of which 40 to 45 million are completely blind and the rest more or less weak-sighted. Regenerative diseases affecting the retina are the

leading cause of blindness worldwide in the elderly. (Finnish Federation of the Visually Impaired 2011; WHO 2011)

2.3.1. Age-related macular degeneration

AMD is the most common cause of blindness among people over 60 years of age. In industrialised countries, clinical signs of AMD are detected at least in one third of persons over 75 years. In Finland, every tenth person over 60 years of age suffers from AMD (Finnish Federation of the Visually Impaired 2011), and the disease is estimated to affect over 30 million people worldwide out of which 14 million are blind or severely visually impaired. (Gehrs et al. 2006)

The AMD is an ocular condition where the central area of retina known as macula is damaged. The AMD occurs either as a consequence of neovascularization or from cell loss due to the geographic atrophy. The first is more commonly referred to as wet form of AMD and the latter as dry form. The AMD is generally thought to progress from the dry form into the wet one with approximately 10 to 15% of AMD patients finally developing the wet form. The AMD does not usually show clinical marks before the age of 55. Clinical signs of dry AMD are pigment disruption and formation of drusen in between RPE and BM. In addition, geographic atrophy and RPE changes are also seen in dry AMD. The clinical signs of wet AMD include fluid, exudates and blood in the extracellular space between the neural retina and the RPE. The wet form of AMD is a more rapidly progressing form and it may result in a sudden loss of central vision. (Gehrs et al. 2006; Nowak 2006)

The therapeutic approaches for AMD have focused mainly on the wet form of AMD due to its rapid progression and debilitating nature. However, there is neither prophylactic therapy nor a cure for the disease. The current treatments aim to avoid further vision loss rather than to improve vision. (Gehrs et al. 2006) The most promising results have been gained with anti-VEGF therapy with reasonable proportion of patients showing vision improvements. However, these anti-VEGF treatments require monthly injections and visits to physicians. (Nowak 2006; Miller et al. 2010)

2.3.2. Retinitis pigmentosa

Retinitis pigmentosa (RP) is a family of inherited heterogenous retinal diseases which are characterized by progressive RPE degeneration and photoreceptor apoptosis. In fact, the RP is the most common inherited retinal disorder with approximately 1.5 million persons affected worldwide. The clinical presentation of RP may alternate, but in general it leads to the progressive degeneration of the photoreceptors causing problems such as night blindness and visual field defects. The RP includes several disorders with diverse chromosomal, metabolic and morphologic findings, of which many are genetically predetermined. (Shintani et al. 2009) Most of the damaged genes that have been discovered concern the rod photoreceptors (Kolb 2007). Clinical findings in RP include arterial narrowing, hyalinization, and waxy optic disc pallor. Changes in

pigmentation of RPE and migration of RPE into retina are also found. RP usually affects one eye, but cases where both eyes are damaged have also been reported. (Shintani et al. 2009; Natarajan 2011) As for AMD, there is no cure for RP. The research of treatment for RP is focused on investigational therapies such as gene therapy to correct the specific mutations, cell transplantation to replace the lost cells and pharmacologic substances to preserve the photoreceptors. (Shintani et al. 2009)

2.4. Transplantation of RPE cells

In many cases of retinal degeneration, the inner layers of the retina and associated neural and glial cells and the optic nerve remain their functionality and organized structure for a period of time. Therefore, by introducing a population of healthy cells, with the capacity to promote photoreceptor survival, into the damaged subretinal area, the vision could be restored. (Hynes et al. 2010) Transplantation of healthy RPE cells will not only fill in the cells lost during degeneration, but RPE also secrete ECM proteins and PEDF, which possess antiangiogenic properties, and VEGF. Most of the RPE transplantation studies in humans have focused on the treatment of wet AMD. (da Cruz et al. 2007)

Adult and fetal RPE cells have been transplanted into the subretinal space of several animal models as well as into humans. Autologous cells, genetically modified RPE cell lines, homologous and heterelogous RPE cells as suspensions or as monolayers on substrates have been investigated in transplantation purposes. (Algvere et al. 1999; Cruz et al. 2007; Sheridan et al. 2009) The feasibility of mature adult RPE cells in AMD has been demonstrated by autologous transplantation of RPE cells from the periphery of the patients own RPE (Binder et al. 2002). Even though the mature adult RPE cells might survive after transplantation to some extent, they often fail to exhibit widespread integration with host retina and end up forming irregular rosette-like structures instead of monolayers (Hynes et al. 2010).

HPSC-derived RPE cells are considered to be a potential cell source for transplantation purposes for dry form of AMD. The use of human embryonic stem cell (hESC)-derived RPE cells could be used in the treatment of the dry form of AMD, where the BRB is intact and the blood cells cannot enter the subretinal space. (Sheridan et al. 2009; Hynes et al. 2010) The first clinical trials with hESC-derived RPE cells for the treatment of advanced dry form of AMD are ongoing. The phase I and II clinical trials are conducted in United States by Advanced Cell Technology (ACT). Recently, ACT got an approval to start clinical trials in United Kingdom and United States with hESC-derived RPE cells to treat Stargardt's macular dystrophy, which is the most common form of inherited juvenile macular degeneration. (ACT 2011)

The delivery of cell suspensions into the subretinal space usually results in poor cell survival. Furthermore, the organization of transplanted RPE cell suspensions and tissue is abnormal and the monolayer structure of intact RPE with transplanted cell material is yet to be gained. RPE transplantations with scaffolds have shown

improvements in RPE cell survival and organization. The scaffolds provide the cells structural support, aid in cell delivery, direct cell behaviour and can also deliver drugs or growth factors. (Hynes et al. 2010) An ideal scaffold for RPE transplantation should be biocompatible and extremely thin, 5 to 90 μm, in order to fit the subretinal space. Furthermore, the scaffold material should be implantable by a minimal invasive surgery technique. The material must also be mechanically durable and flexible to withstand surgical manipulation and avoid harming surrounding tissue. In addition, the material must have good permeability or porous structure in order to have sufficient delivery of nutrients and other substances. Several scaffold materials for RPE cell transplantation has been tested. (Hynes et al. 2010) For instance, collagen, PLLA, PLGA, gelatine, poly(glycerolsebacate), poly(methylacrylate/methacrylamide), poly(dimethylsiloxane) (PDMS) and polyurethanes have been studied as possible scaffold materials for RPE cell transplantation (Giordano et al. 1997; ; Binder et al. 2007; Hynes et al. 2010).

3. STEM CELLS

3.1. Human embryonic stem cells

The discovery of the cells which are able to divide indefinitely and differentiate into specialized cell types has opened new avenues for regenerative medicine (Vazin et al. 2010). The cells in question are stem cells and they possess an enormous differentiation potential towards somatic cell types. Stem cells are undifferentiated cells, which can divide in two possible ways: symmetrically or asymmetrically. In symmetric division, a stem cell divides into two identical stem cells (Bongso et al. 2004). This process is called self-renewal. In asymmetric division, also called differentiation pathway, one daughter cell remains as a stem cell and the other differentiates into a precursor cell, which proliferates and continues to differentiate towards specialized cell type. The difference between a stem cell and a precursor cell is that stem cell has telomerase activity, which enables the cell to divide without a limit. Telomerase is an enzyme, which adds short DNA sequences, telomeres, to the ends of eukaryotic chromosomes. (Raff 2003)

Stem cells can be classified in two ways, either according to their source or development potential. The fertilized egg, zygote, is a source of totipotent stem cells. ESCs are derived from the inner cell mass (ICM) of a blastocyst. ESCs are pluripotent and they can be expanded *in vitro* and kept in this pluripotent phenotype indefinitely. (Langer et al. 2007) In an adult human body, somatic adult stem cells (ASCs) are found in most tissues and organs. Examples of ASCs are mesenchymal stem cells (MSCs), which are widely spread in the connective tissues, and neural stem cells (NSCs) found in the central nervous system. The limitation with the adult stem cells is that they are sparsely distributed in tissues and difficult to isolate. Stem cells from the foetus have been used in research, as well. (Bongso et al. 2004) The classification of stem cells according to their development potential is also a common way. Totipotent cells have the capability to form all the cell types of an individual and are basically capable of forming the entire organism. The term pluripotency means that those stem cells lack the latter feature, but they are able to produce all the cell types of all three germ layers. The cells producing more than one cell type in tissues are said to be multipotent and those producing only one are unipotent. (Raff 2003)

hESCs are isolated from donated spare embryos of blastocyst stage after *in vitro* fertilization (Thomson et al. 1998). ICM consists of a cluster of 10 to 30 cells inside a five to eight day old embryo. (Bongso et al. 2004) Furthermore, hESCs can be derived from earlier morula-stage human embryos or intact blastocysts, from which the

glycoprotein shell zona pellucida has been removed (Trounson 2006). Colonies of hESCs differ from ICM in many ways: ICM preserves a memory of axes, where as ESCs are capable of indefinite proliferation *in vitro*. The hESC lines also express high levels of telomerase activity. (Thomson et al. 1998) hESCs grow in tightly packed colonies which have defined borders at the periphery of the colonies. Individual hESCs have a high nucleus to cytoplasm ratio and a prominent nucleoli. In addition, hESCs express classical markers of pluripotent cells such as OCT4, nanog, TRA antigens, state-specific embryonic antigen-4 (SSEA-4), SSEA-3 and alkaline phosphatase. (Vazin et al. 2010) Conventional way of testing the pluripotency of hESCs is teratoma formation as a xenograft to immune-compromised mice. A teratoma is an encapsulated tumor with cells or tissues representing all the three germ layers of the embryonic disc (Ergec et al. 2009). hESCs exhibit many differences from mouse embryonic stem cells (mESCs): the growth of hESCs is slower and they tend to form flat colonies instead of spherical ones. First hESC lines were derived by Thompson et al. in 1998. (Thomson et al. 1998)

Nowadays several protocols for derivation of hESC-lines have been established and also numerous studies of culturing undifferentiated hESCs and their differentiated progeny have been performed (Stojkovic et al. 2004). To date, isolation of the ICM from blastocysts has been achieved for the most part by immunosurgery or mechanical dissection. The first few hESC lines were established using the immunosurgical method. However, this procedure requires the use of animal-derived products, such as anti-human serum antibodies and guinea pig complement, which may prevent the later use of these hESCs in transplantation therapies due to the possible transfer of pathogens into patients. Therefore, the mechanical or enzymatical dissociation of ICM in such manner that avoids the use of animal-derived products during the derivation process would be advantageous from the clinical point of view. (Vazin et al. 2010)

The first hESC lines were cultured on mouse embryonic fibroblast (MEF) feeder layers, which support the propagation of hESCs in undifferentiated state. However, in order to move towards xeno-free hESC culture systems, several approaches exploiting human-derived cell types such as fibroblast feeder cells derived from fallopian tube epithelium, fetal foreskin, muscle, bone marrow, or amniotic epithelium. Optionally, hESCs can be cultured in feeder-free cultures in the presence of extracellular matrixes such as Matrigel^{TM} and fibronectin. Medium conditioned by feeder fibroblast cells and supplemented with bFGF has been initially used to maintain hESCs in undifferentiated state in feeder-free environment. (Vazin et al. 2010)

Despite the fact that hESCs maintain their pluripotent nature, it is evident that hESCs can acquire karyotypic abnormalities during prolonged maintenance *in vitro*. These chromosomal deviations are thought to be a result of *in vitro* adaptation and selection since abnormal rapidly-dividing cells step by step predominate and take over the entire culture. (Baker et al. 2007) Therefore, the hESC cultures intended for therapeutic use are regularly karyotyped with traditional Giemsa-staining, which detects large chromosomal changes, or with other methods such as fluorescent *in situ* staining,

DNA microarray, whole-genome single nucleotide polymorphism, and short tandem repeat analysis. (Vazin et al. 2010)

3.2. Induced pluripotent stem cells

The use of hESCs in therapeutic purposes in regenerative medicine arouses ethical concerns. Moreover, the possible tissue rejection following transplantation of hESCs into patients constricts the use of hESC as a therapeutic tool. Several strategies to induce pluripotency in already mature somatic cells has been conducted, such as somatic cell nuclear transfer and fusion of somatic cells with ESCs, in hopes of generating patient-specific cells of any lineage. However, these protocols confronted technical, ethical and logistical barriers that restricted the use of resulting pluripotent cells in both research and clinical therapy. (Maherali et al. 2008) The idea of using patient's own cells and generating them into induced pluripotent stem cells (iPSCs) took a huge step forward in 2006 by Takahashi and Yamanaka, who reprogrammed mouse embryonic and adult fibroblast cultures to pluripotent stem cells by retroviral delivery of four transcription factor genes; sex-determining region Y- box 2 (*SOX2*), octamer 4 (*OCT4*), Krueppel-like factor 4 (*KLF4*) and *C-MYC*. The research of Takahashi et al. was groundbreaking for the field of regenerative medicine; already mature somatic cells were reprogrammed back to pluripotent cells with a large capacity to differentiate into mature cell types without the use of embryonic material. (Takahashi et al. 2006) The first hiPSC lines were derivated a year later by Yu et al. and Takahashi et al. from human fibroblasts. Takahashi et al. reprogrammed the cells with same four transcription factors as reported in their pioneer work with iPSCs, whereas Yu et al. showed that reprogramming of human induced pluripotent stem cells (hiPSCs) can be induced by *SOX2*, *OCT4*, *NANOG* and *LIN28*. (Yu et al. 2007; Takahashi et al. 2007)

Properly reprogrammed iPSCs possess wide range of properties including loss of somatic cell-specific markers, expression of appropriate stage-specific embryonic antigens, telomerase activity, X chromosome reactivation in female cells, reactivation of endogenous genes for pluripotency and self-renewal such as *SOX2*, *OCT4* and *NANOG*, and silencing of exogenous factors for the initiation of reprogramming. Moreover, iPSCs are able to self-renew and contribute to all three germ layers during teratoma formation similar to ESCs. (Cox et al. 2010) Morphologically, the iPSCs appear identical to ESCs. Also, human iPSCs as well as ESCs survive poorly as single cells. Therefore, the initial passaging of new colonies must be done mechanically and several passages are required before the cells can be adapted to enzymatic dissociation. (Maherali et al. 2008) In addition, hiPSCs seem to require an FGF, such as FGF2, to maintain their pluripotency and self-renewal capacity in culture conditions. Interestingly, gene expression profiling has revealed slight differences between hESC lines and iPSC lines. (Cox et al. 2010) Furthermore, the general assumption that autologous iPSCs are not immunogenic might need more inspection. The study of Zhao et al. in mice suggests that abnormal gene expression in some cells differentiated from

iPSCs can induce T-cell-dependent immune response in genetically identical recipients. Therefore, the immunogenicity of therapeutic cells derived from patient-specific hiPSCs should be examined before transplanting these cells into patients. (Zhao et al. 2011) Figure 7 illustrates the reprogramming of somatic cells into pluripotent stem cells.

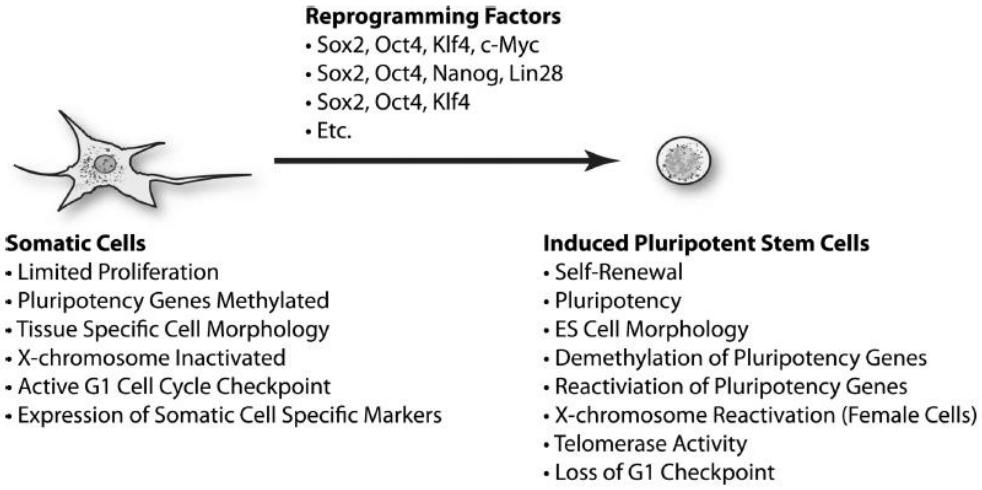

Figure 7. *Reprogramming mature somatic cells to iPSCs. (Cox et al. 2010)*

Since the first reprogramming by Takahashi and Yamanaka, it has become clear that there is some flexibility in the factors required for reprogramming somatic cells. For example, c-Myc is not strictly needed for the reprogramming of somatic cells. However, when c-Myc is left out from the reprogramming factors, the time required for the protocol is significantly longer, and the efficiency of the protocol decreases dramatically. Human fibroblasts have also been reprogrammed using a gene combination of *SOX2*, *OCT4*, *LIN28* and *NANOG*. Alternatively, some reprogramming factors can be replaced by transcription factors from the same family. In general, the specific factors required for reprogramming depend on the somatic cell type being reprogrammed. (Cox et al. 2010)

A variety of methods exist for iPSC derivation (Maherali et al. 2008). Several reprogramming protocols are based on retroviruses or lentiviruses, which integrate into the genome of somatic cells and express the exogenous reprogramming factors. However, the continuous expression of reprogramming factors and the possibility for insertional mutagenesis are justified concerns and risks which complicate the clinical use of hiPSCs. (Cox et al. 2010) Therefore, reprogramming protocols in which the genetic material would not permanently integrate into the genome have been developed. One approach has been a protocol in which the genetic material is first integrated into the genome and later removed. In these protocols, after the viral integration and reprogramming, recombinases were used for removing the reprogramming factors from the genome. (Soldner et al. 2009; Kaji et al. 2009) In addition, non-nucleic acid based rerprogramming protocols have been investigated. For example, purified recombinant proteins of transcription factor genes containing a polyarginine protein transduction domain in their C-termini resulted in iPSC formation after 30 days after the initiation of

reprogramming. (Zhou et al. 2009) Furthermore, the use of small molecules, such as valproic acid and 5-aza-cytidine, has been shown to result in fully reprogrammed iPSCs (Huangfu et al. 2008; Mikkelsen et al. 2008).

3.3. Culturing hPSCs in feeder-free conditions

Culturing undifferentiated hPSCs for a long period of time *in vitro* is traditionally carried out on fibroblast feeder cell layers. However, using feeder cells in hPSC cultures is quite vulnerable and limits the production of hPSCs in larger amounts for the possible use in regenerative medicine. The production of feeder cells is laborious. In addition, the goal towards xeno-free hPSC cultures and clinical applications restricts the use of animal-derived materials, such as fetal bovine serum (FBS), commonly used in fibroblast feeder cell propagation. In addition, the function of feeder cells in hPSC cultures is not yet fully unravelled. To date, it is evident that feeder cells provide hPSCs essential cell-cell contacts as well as vital soluble factors for maintaining the hPSCs in undifferentiated state. (Hakala et al. 2009) Feeder-free cultures would be free of many challenges related to feeder cell layers.

The first cultures of hESCs in feeder-free conditions were created by replacing the feeder cells with Matrigel™. Matrigel™ is an ECM product secreted by mouse Engelbreth Holm-Swarm sarcoma cells, which contains mainly laminin, collagen IV, heparin sulphate proteoglycan, growth factors and other substances. For example, hESCs have been cultured on Matrigel™ in MEF-conditioned media. In these conditions, hESCs grew as dense and undifferentiated colonies, but they were surrounded by differentiated cells in between the colonies. (Xu et al. 2001) In addition, Matrigel™ has been used as a culture substrate for hESCs by several other research groups with supplements such as conditioned media (CM) and serum replacements (SR) (Xu et al. 2004; Sato et al. 2004; Xu et al. 2005; Wang et al. 2005). Besides Matrigel™, other ECMs have been used for maintaining hESCs in undifferentiated state in feeder-free conditions. For instance, laminin, fibronectin and combination of collagen IV, fibronectin, laminin and vitronectin have been investigated with in combination of non-defined and defined culture mediums (Xu et al. 2001; Xu et al. 2004; Noaksson et al. 2005; Ludwig et al. 2006a; Hakala et al. 2009). However, all the research groups who have conducted comparative studies with ECMs agree that animal-derived Matrigel™ is a superior matrix for maintaining hESCs in feeder-free conditions (Xu et al. 2001; Xu et al. 2004; Hakala et al. 2009).

For mouse embryonic stem cells (mESCs), the feeder cell layer can be replaced by the addition of leukaemia inhibitory factor (LIF) to the culture medium. However, this approach is not effective for hESCs. (Thompson et al. 1998) For hESCs, bFGF is the most commonly used medium component required for hESC maintenance with or without feeder cells. (Xu et al. 2001) Significant concentrations of bFGF can support long-term feeder-independent culture of hESCs when supplemented with noggin (Xu et al. 2005). Furthermore, supplements such as TGFβ, activin A., keratinocyte growth

factor, nicotinamide and sphingosine-1-phosphate/platelet-derived growth factor (PDGF) have been shown to improve the survival of hESC cultures (Chase et al. 2007). Recently, many publications have described defined xeno-or feeder-free media formulations for the maintenance of hESCs (Vallier et al. 2005; Lu et al. 2006; Ludwig et al. 2006a; Ludwig et al. 2006b; Wang et al. 2007). Ludwig et al. developed a serum and animal protein free medium called TeSR™ that supports derivation and long-term culture of hPSCs in feeder-free conditions. However, hESC lines derived with TeSR™ were found to be karyotypically abnormal. (Ludwig et al. 2006a) Therefore, Ludwig and his colleagues modified the TeSR™ medium into a more economical version, called mTeSR®1, which includes some animal-sourced proteins, such as bovine albumin source. mTeSR®1 possess the advantage of being fully-defined and serum-free, and it can be used in combination with Matrigel™. (Ludwig et al. 2006b)

In feeder-free conditions, hESCs often give rise to fibroblast or stromal-like cells that may support the maintenance of undifferentiated growth of hESCs. In fact, few research groups have indicated that these feeder cells derived from hESCs can support their own growth. (Stojkovic et al. 2005; Wang et al. 2005) However, these cells are not immortal and they will senescence after several passages, which limit their continuous use. Furthermore, the derivation process of these feeder cells from hESCs can be difficult and may result in variable culture conditions. (Vazin et al. 2010)

Future efforts are necessary in order to develop a totally xeno-free and defined culture method for stem cells which maintains the self-renewal capacity and proliferation of hPSCs. The current attempts usually involve some animal-based substances or result in chromosomal abnormalities and instability therefore limiting the therapeutic use of hESCs. In addition, lower cost alternatives are necessary to make use of the developed system for routine research. (Chase et al. 2007)

3.4. Differentiation of hPSCs into RPE cells

The enormous need for cells that could be used in cell transplantation therapy has driven the researchers to find appropriate cell sources for RPE. Particularly hESCs are considered to be a potential cell source for RPE transplantation due to the anatomical location of RPE in the BRB. This localization of RPE layer in the inner surface of the BRB in the subretinal space separates the transplanted cells from blood circulation and therefore is out of reach for the immuno system and immune rejections in dry form of AMD.

Several approaches have been studied in order to differentiate hPSCs into RPE cells. The first cells with RPE appearance were identified from primate cultures in the beginning of last decade. Kawasaki et al. used a protocol based on stromal cell-derived inducing activity (SDIA) from mesenchymal bone marrow cells (PA6 cells) to derive pigmented Pax6 positive cells. The first pigmented cells were detected after 3 weeks of co-culture with PA6 cells. Also Ooto et al. applied SDIA in RPE induction from mouse and primate ESCs. (Kawasaki et al. 2002; Ooto et al. 2003) Later, Aoki et al. showed

that Wnt2b signalling increases the production of RPEs from mESCs with SDIA, whereas Ueno et al. modified the SDIA protocol by discarding the PA6 cells and replacing them by matrix layers of human amniotic membrane. (Aoki et al. 2006; Ueno et al. 2006) However, in all of these studies which applied SDIA protocol lentoid bodies were seen.

Klimanskaya et al. derived RPE from hESCs by overgrowing them on MEF feeders. The hESCs colonies were allowed to overgrow on MEFs until the colonies had lost their tight borders and became multilayered, after which bFGF was removed from the culture media. With this approach pigmentation appeared 2 to 6 weeks after starting the differentiation. However, only small part of the hESCs differentiated into RPE during the period of 3 to 8 weeks. Pigmented areas were selected by hand picking and plated on gelatin-coated culture dishes. After transferring pigmented RPEs on gelatin, they lost their pigmentation and morphology, and migrated along the culture dish. However, when the dish became confluent, the pigment and morphology were regained. (Klimanskaya et al. 2004) The overgrowth protocol of hESCs on MEFs developed by Klimanskaya et al. was reproduced by Lund and his colleagues with similar findings (Lund et al. 2006). The protocol based on overgrowth of hESCs has also been studied by Vugler et al. However, in their protocol, they plated the pigmented cells on Matrigel^{TM} instead of gelatin. (Vugler et al. 2008)

Embryoid body (EB) formation without the overgrowth step on feeders has also been studied for RPE differention. EBs are 3-dimensional (3D) cell aggregates, which give rise to all the three layers of the embryonic disc. Klimanskaya et al. trypsinized hESC colonies and transferred them into EB medium described above and into a low adherence culture plate. Less than 1% of EB-like cell clusters developed pigment in time course of 4 to 8 weeks. However, over the course of 6 to 9 months the cells on the surface of EB-like clusters became pigmented. (Klimanskaya et al. 2004) RPE differentiation via EB-like protocols has also been studied by others. In these experiments, the EB-like clusters were cultured in suspension from 1-3 weeks up to 9 months. After culturing the EBs in suspension for a while, they were seeded on adherent culture dishes with coating, such as laminin. (Meyer et al. 2009; Idelson et al. 2009; Nistor et al. 2010)

Most of these introduced RPE differentiation protocols involved the use of undefined and animal-derived components such as bovine serum albumin (BSA) or MEFs. The first step towards a defined xeno-free differentiation protocol of RPE was taken by Idelson et al. who cultured hESCs on human foreskin fibroblasts (hFFs) instead of MEFs in KO-SR medium. (Idelson et al. 2009) Vaajasaari et al. took this approach further by successfully differentiating hPSCs into RPE using xeno-free and defined culture medium RegES as a base (Vaajasaari et al. 2011).

Numerous factors have been studied for the induction of RPE differentiation. These include Wnt antagonists, nicotinamide, neural induction medium with N2 supplement and heparin or B27, activin A and TGF-β1. (Meyer et al. 2009; Idelson et al. 2009; Lu et al. 2009) Despite several attempts to develop a directed differentiation

protocol for RPE from hESCs, efficient protocols have not yet been established. The differentiation is not well defined and only small yields of RPE cells are achievable. Therefore, the ideal factors to induce direct differentiation are still being vigorously investigated. (Rowland et al. 2011)

4. MICROPARTICLES

Microparticles have diameters ranging from 1 to 500 μm (Figure 8). Microparticles were used in cell culture studies for the first time in 1967 by van Wezel, who noticed that microparticles facilitated the growth of mammalian cells. (van Wezel 1967) Since then, the microparticles have been successfully used in the growth of anchorage-dependent cells for the production of vaccines and pharmaceuticals, and to expand cell populations. Microparticles offer the advantage of providing a larger surface area for the growth of anchorage-dependent cells in a suspension cultures, and overcome several issues with static cultures, such as the need for large quantities of culture media, space expense, inefficient gas-liquid oxygen transfer, presence of concentration gradients and difficulties in monitoring and control. (Martin et al. 2011; Sun et al. 2011) In the next chapters, the preparation methods and modifications of microparticles and their use and potential in cell culture and tissue engineering are introduced.

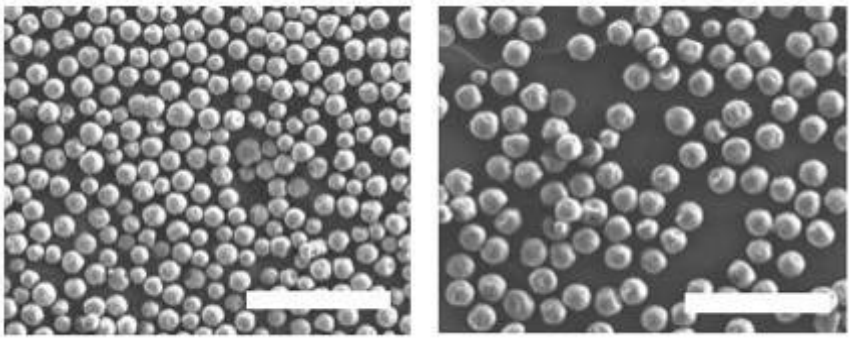

Figure 8. *Chitosan microparticles. Scale bar 200μm. (Modified from the source Liu et al. 2011)*

4.1. Preparation of microparticles

The preparation method for microparticles determines the characteristics of the prepared particles, including their size. Therefore, knowledge of the experimental parameters such as solvents, temperature, stabilizers and stirring rate in preparation protocols are vital. Even more crucial is to know how these parameters cause changes in the characteristics of resulting particles. For instance, methods based on emulsions, nanoprecipitation, salting-out and spray-drying have been used in microparticle preparation. (Lassalle et al. 2007)

4.1.1. Emulsion-based methods

In emulsion-based methods the solvent, where the polymer is dissolved in, is eliminated. The elimination of the solvent can be conducted either by evaporation or by extraction. The emulsion can contain aqueous and oily phases. Usually, the polymer is incorporated in to the organic phase, and the emulsifier is contained in the aqueous phase. By mixing the two phases, an emulsion is formed. As the solvent evaporates, the microparticles are formed. (Lassalle et al. 2007)

In a simple emulsion, a stabilizing agent is in the aqueous phase. Double emulsions are modifications from single emulsions in which the delivered active emulsifier is dissolved in an aqueous phase and the polymer is dissolved in an organic phase. These solutions are combined and a single emulsion is formed. The formed emulsion is then poured into the aqueous media that includes the stabilizing agent after which the solvent is removed, and microparticles are solidified. The solvents used for polylactide (PLA) and polylactide-co-glycolide (PLGA) need to be immiscible with the emulsion solvent. The most commonly used solvents are ethyl acetate and dichloromethane (DCM). On the other hand, the function of the emulsifier is to form a thin layer around the oil drops and polymer reducing the coagulation of the polymer drops and thus stabilize the emulsion. (Lassalle et al. 2007) For instance, Bible et al. prepared PLGA microparticles with average particle size between 50-100 μm by a single emulsion method. They used polyvinylalcohol (PVA) as a stabilizer and the polymer was dissolved in DCM. (Bible et al. 2009)

4.1.2. Nanoprecipitation

Nanoprecipitation is based on interfacial deposition of polymers, which is followed by a displacement of a semi-polar solvent miscible with water from a lipophilic solution. It is a rather easy and reproducible technique that has been used in the preparation of nano- and microparticles. Nanoprecipitation does not require large amounts of toxic solvents. Nevertheless, the main problem with the nanoprecipitation method is the frequent agglomeration of the particles due to the absence of a stabilizer. Furthermore, removal of solvent residuals from the prepared nano-and microparticles is rather difficult. (Lasselle et al. 2007)

4.1.3. Salting-out

An alternative method for the widely applied emulsion procedure is salting-out. This method includes the use of a solution in which the polymer is dissolved and a water-miscible solvent such as acetone or tetrahydrofuran. The solution is emulsified under vigorous stirring in an aqueous gel that contains the salting-out agent and a stabilizer, if required. Addition of large amount of water to the emulsion leads to the formation of spheres, which are purified and recovered by cross-flow filtration. The salting-out agents are usually electrolytes, such as magnesium chloride, sodium chloride and

magnesium acetate. However, also non-electrolytes, such as sucrose, have been used. Salting-out is a suitable method for microparticle preparation when high quantities of polymer are applied. Nevertheless, the need for extensive purification of the prepared particles limits the use of salting-out method. Furthermore, most of the salts used as salting-out agents are incompatible with bioactive compounds. (Quintanar-Guerrero et al. 1998; Lassalle et al. 2007)

4.1.4. Spray-Drying

Spray-drying method is quite often used in microparticle preparation. In this particle preparation method, the polymer is dissolved into an organic solution which is then sprayed into a hot-air flow. The solvent instantly evaporates in the hot air, and dry microparticles can be collected. In spray-drying procedure, organic solvents can be avoided. In contrast to the emulsion-based techniques, the spray-drying is a very rapid procedure, which makes it scalable for industrial levels. However, a major limitation with spray-drying is a non-uniform particle size. In addition, there might also be significant loss of product during the process due to the adhesion of microparticles to the walls of the spray dryer or the aggregation of the particles. (Lassalle et al. 2007)

As a consequence of these limititations with conventional spray-drying method, novel approaches based on the spray system have been developed. For instance, in ultrasonic atomization the atomized polymer dispersion was sprayed into a non-solvent where the polymer solvent was extracted. As a result, microparticles were formed. (Felder et al. 2003) Liu et al. prepared chitosan microparticles with uniform size and morphology by a novel microfluidic jet spray drier. They also reported that the particle size could be altered by changing the concentration of chitosan solution. (Liu et al. 2011) Alhnan and his colleagues produced pH-responsive microparticles by spray-drying aqueous polymer solutions and Yeo and coworkers developed a spray-drying system where the production of microparticles occurs on the surface of an aqueous droplets generated by a dual microdispenser system. In the latter one, the microparticles are formed through the collision of two droplets of the aqueous and the polymer solution. (Yeo et al. 2004; Alhnan et al. 2011)

4.2. Commercial microparticles

Microcarriers are composed of a stabile matrix that can withstand mechanical forces for example in a perfused bioreactor. Today, a vast number of microparticles are in use and also commercially available. For instance, microparticles composed of glass, polystyrene, collagen, gelatine and dextran with different coatings are available for research purposes. Furthermore, microparticles with different coatings and treated surfaces are available in order to improve the attachment of anchorage-dependent cells. Also, a large number of particles with different size distributions are on the markets. (Martin et al. 2011; Sun et al. 2011) Examples of commercial microparticles that are available for research purposes are listed in Table 1.

Table 1. *Examples of commercial microcarriers and their special features available for research purposes. (Nedovic et al. 2004; Oh et al. 2009; Chen et al. 201; Martin et al. 2011; Sun et al. 2011)*

Microparticle	***Manufacturer***	***Material***	***Special feature***	***Charged group***
Biocarrier	Bio-Rad	Polyacrylamide	Positive charge	Dimethyl-aminopropyl
Biosilon™	Nunc	Polystyrene	Negative charge	-
Cellagen™	MP Biomedicals	Collagen	-	-
Cultispher™-G	Percell Biolytica	Gelatin	Macroporous	-
Cultispher™-S	Percell Biolytica	Gelatin	Macroporous	-
Cytodex™ 1	GE Healthcare	Dextran	Positive charge	Diethylaminoethyl
Cytodex™ 3	GE Healthcare	Dextran	Gelatin coating	
DEAE	Sigma	Cellulose	Positive charge	Diethylaminoethyl
DE 53	Whatman	Cellulose	Positive charge	Diethylaminoethyl
Hillex™	Solohill	Dextran	Positive charge	Cationic trimethyl ammonium
HyQSphere™	Solohill	Polystyrene	-	-
QA52	Whatman	Cellulose	Positive charge	Quaternary ammonium
SphereCol®	Advanced Bio matrix	Collagen	-	-
Tosoh 65PR	Tosoh Bioscience	Hydroxylated methacrylate	-	Primary amine

4.3. Biodegradable microparticles

Despite there is a vast supply of commercial microparticles available, most of these are not that useful in tissue engineering or direct transplantation because they do not decompose in the human body. Majority of the commercial biodegradable microparticles are composed of gelatin or collagen, which are usually derived from different species. Therefore they are animal-derived and might contain potential pathogens. Furthermore, trypsin, collagenase and dispase have been used to retrieve viable cells from biostabile microparticles for use in cell-based transplantation studies with rather poor success. The cells separated from the beads imperfectly after enzymatic treatment. Therefore development of the biodegradable microparticles that allow the cells to detach when the particles degrade could be an ideal solution. (Sun et al. 2011) Biodegradable polymers are capable of degrading into biocompatible byproducts via

chemical or enzyme-catalyzed hydrolysis. The biodegradation of specific biomaterials makes it possible to implant them into the body without the need of removal of the implant by surgical operation. Biodegradable microparticles are very useful because they can be administered to a variety of locations *in vivo* by a syringe needle. (Park et al. 2004) In the following chapters, several potential biodegradable polymers for microparticle preparation and applications are introduced.

4.3.1. Biodegradable polymers for microparticles

Biodegradable polymers such as polyesters, polyanhydrides, poly(orthoesters), polyphosphazenes and polysaccharides have been used in preparation of microparticles. Especially aliphatic polyesters have attracted interest as potential microparticle materials due to their biocompatibility and biodegradability. (Park et al. 2004)

Polyesters are synthetic polymers which degrade via hydrolytic degradation of the ester bonds in their backbone. PLGA is probably the most widely used aliphatic polyester in microparticle preparation. The huge popularity of PLGA among researchers is based on its adjustable degradation rate and mechanical properties, which can be controlled by varying the lactic acid/glycolic acid ratio and by changing the molecular weight of the polymers. PLGA polymers are less stiff than the original polyglycolide (PGA) polymers. The PLGA microparticles undergo bulk degradation, in which the polymer matrix preserves its original shape and mass until significant degradation has occurred. As the molecular weight of PLGA decreases, the degradation rate increases. (Park et al. 2004; Nair et al. 2007) The properties of PLGA vary naturally according to the lactide and glycolide ratios. Amorphous polymers are obtained with a lactide/glycolide monomer ratio of 25:75 whereas a copolymer with a monomer ratio of 80:20 is semicrystalline. When the L-lactide/glycolide-ratio of the copolymer increases, the degradation rate of the copolymer decreases due to the more crystalline structure of the polymer. By changing the lactic acid to glycolic acid monomer ratios, the degradation time can vary from 1-2 to 5-6 months. Furthermore, the lactid acid is a chiral molecule, and exists in two optically active forms; L-lactide and D-lactide, of which the L-lactide is the naturally occurring form. These optic isomers have an effect on the properties of PLGA copolymers, such as crystallinity, as well. (Park et al. 2004; Nair et al. 2007; Vroman et al. 2009)

Other poly(α-esters), PLAs, have proven to be useful in biomedical applications due their biodegrability and non-toxicity (Lasselle et al. 2007). The copolymerization of L-lactide with D-lactide produces copolymers with different degree of crystallinization depending on the monomer ratio used. For example, poly(L-lactide) (PLLA) is a semicrystalline polymer with rather low glass transition temperature and quite slow degradation rate, whereas poly(DL-lactide) is an amorphous polymer. (Vroman et al. 2009) With high molecular weight PLLA, the total degradation can take 2 to 5.6 years *in vivo.* The degradation mechanism of PLLA is bulk erosion. Compared to PLGA, PLLA has an extra methyl group in its molecular structure. This methyl group increases the hydrophobicity of PLLA. Due to a more hydrophobic nature, PLLA has

significantly slower degradation time compared to PLGA. In addition, PLLA is more resistant to hydrolysis due to the steric shielding effect of the methyl side groups. PLLA has also good mechanical properties with good tensile strength, low extension and a high modulus. Thus, PLLA is considered to be an ideal biomaterial for load bearing applications. (Nair et al. 2007) Chemical structures of common polyesters used in microparticle preparation are illustrated in Figure 9.

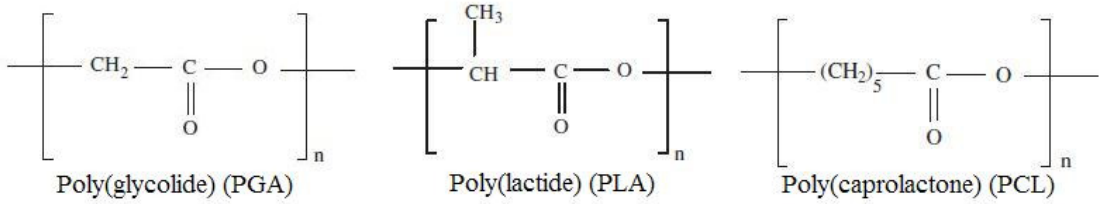

Figure 9. *Chemical structures of biodegradable polyesters used in microparticle preparation. (Modified from the source Nair et al. 2007)*

Besides PLGA and PLA, poly(ε-caprolactone) (PCL) microparticles have been prepared. PCL is biodegradable, semicrystalline polymer with low glass transition temperature. It is quite hydrophobic polymer, which in combination to crystalline structure leads to quite slow degradation rate of 2 to 3 years. Therefore PCL is suitable for long-term applications. The PCL devices also undergo hydrolytic degradation. (Nair et al. 2007; Park et al. 2004)

Eventhough several synthetic biodegradable polymers have been developed for biomedical applications, the use of natural biodegradable polymers remains attractive because their abundance in nature, good biocompatibility, and processability. Proteins, such as collagen, gelatin and albumin, have been studied and used in microparticle preparation in addition to polysaccharides, such as starch, dextran, hyaluronic acid and chitosan. However, protein-based natural polymers have poor mechanical properties, low elasticity, possible immunogenic parts and high cost. Instead, microparticles composed of polysaccharides are commercially available at low costs and broad range of physicochemical properties. For example, chitosan shows excellent biocompatibility, biodegradability and low immunogenicity. Chitosan contains primary amino groups in the main backbone that makes the surface of prepared microparticle positively charged. (Park et al. 2004; Nair et al. 2007)

4.3.2. Pharmacologically active microparticles

Biodegradable polymers allow incorporation of drugs, which can be released in a controlled manner. The release rates of the drugs from biodegradable polymers can be controlled by biodegradation kinetics of the polymers, physicochemical properties of the polymers and the shape of the devices. A variety of drugs can be loaded into these particles. (Park et al. 2004) Pharmacologically active microparticles (PAMs) are

particles that are able to convey cells on their surface and release a growth factor or other pharmacologically active substance simultaneously. The released agent can improve the survival, differentiation or integration of cells into host tissue in transplantation. The pharmacological agent can also treat the surrounding tissue, therefore acting as a drug delivery device. (Tatard et al. 2007)

In order to improve the outcome of foetal dopaminergic cell transplantation for the treatment of Parkinson's disease, Tatard et al. incorporated a glial cell-derived neurotrophic factor (GDNF) into PLGA microparticles. The effects of implantating the foetal dopaminergic cells on PAMs were investigated in hemiparkinsonian rats. The transplantation of the cells on PAMs resulted in improved cell survival compared to the cells transplanted as suspension. Furthermore, the cells had more neuritis with GDNF releasing PAMs than with unloaded PAMs. (Tatard et al. 2007) PAMs have also been studied in *in vivo* differentiation of cartilage from MSCs. Bouffi et al. incorporated a chondrogenic differentiation factor TGF-β3 into PLGA microparticles in single emulsion method. Human MSCs attached rapidly onto the fibronectin coated PAMs, and strong up-regulation of cartilage-specific cell markers was observed after 3 weeks cultivation. Moreover, these TGF-β3 containing microparticles resulted in histologically resembling cartilage in mice. (Bouffi et al. 2010)

4.4. Properties and modifications of microparticles

Many biomaterials as such do not have ideal surface properties for cellular attachment. Therefore, modifications of the particle properties to increase the cell attachment are necessary. Mainly four approaches have been used for microparticle modifications to increase cell attachment. These include coating particles with ECM proteins, changing the overall charge of particle surface, surface treatments to increase the hydrophilicity of the particles and finally varying the polymer composition and porosity. The microparticle surfaces interact with cells mainly by facing ions and low molecular weight organic molecules and proteins. The basic idea behind microparticle modifications is to design surfaces that mimic the properties of ECM. (Nedovic et al. 2004)

4.4.1. Size and shape

The size and shape of microparticles have an effect on cell propagation and resulting yields in microparticle culture systems. The average size and size distribution of microparticles should be narrow to achieve homogenous culture conditions in microparticle suspension cultures. The preferable size, which can provide the best cell growth, depends on the cell type under investigation. In general, the ideal microparticle size is considered to be 100 to 230 μm for microporous particles. This size range is a compromise between the maximation of the surface area, stability of the dispersion and a surface unit per particle. The first two factors favour smaller particle size, whereas the latter requires larger surface area to provide adhesion and proliferation of hundreds of

cells. (Nedovic et al. 2004) The microparticles in research use today are mostly spherical, but also cylindrical microparticles have shown great results in cell culture studies (Oh et al. 2009; Leung et al. 2010; Lecina et al. 2010).

The size of microparticles has been shown to be the main factor that affects the formation and size of aggregates in microparticle suspension cultures. Larger particles with diameters of 100 to 200 µm have resulted in larger aggregates and broader aggregate size distribution, whereas particles with diameters of 10 µm have shown to attach around cell clumps generating smaller aggregates with soft tissue cores. (Lecina et al. 2010) The tight aggregates formed by small microparticles have shown to reduce the cell yields. This reduction probably results from the limited access of nutrients and growth factors to the cells trapped inside the aggregates. (Chen et al. 2011) Furthermore, the microparticle size did not influence on differentiation of hESCs to cardiomyocytes (Lecina et al. 2010). The cylindrical shape of commercial DE 53 microparticles is illustrated in Figure 10.

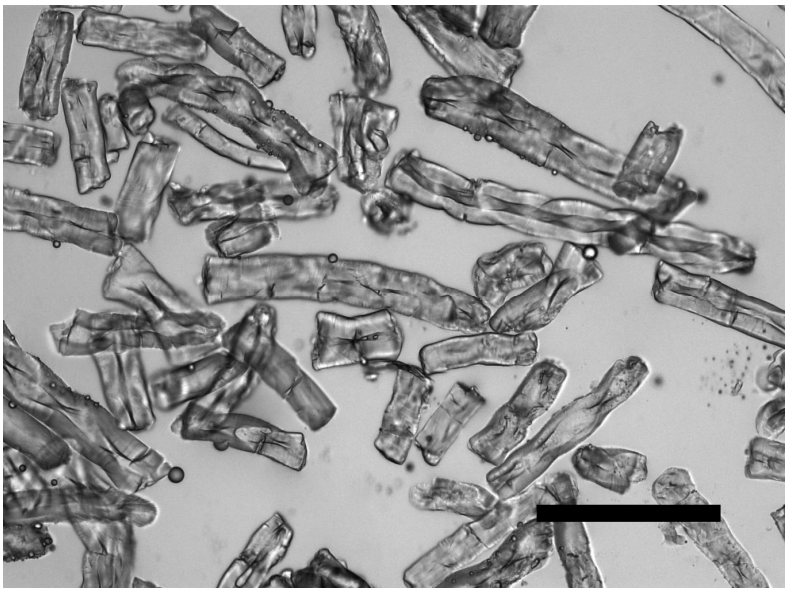

Figure 10. *Commercial DE 53 microcarriers. Scale bar 200 µm.*

A sphere provides the most favourable thermodynamically environment with a reduced surface area to volume ratio. However, this ideal shape does not maximize the surface of the material. Therefore, non-spherical microparticles such as disks made of polyester fibres or particles with cylindrical shape are also in use. Some cell types, such as human colon carcinoma cell line, show better attachment and growth in these anisotropic particles. (Nedovic et al. 2004) A comparison between spherical and cylindrical microparticles with both particle types having similar surface properties has been conducted. In this study, the hESCs on cylindrical particles grew as compact cell-microparticle aggregates, whereas the hESCs on cylindrical particles formed less tight aggregates. Interestingly, the cells on cylindrical microparticles were spread out in thin layers between the microcarriers. However, the shape did not have a dramatic effect on resulting yields and differentiation. (Chen et al. 2011; Lecina et al. 2011) Even though

the size and shape are important parameters, their effects on cell attachment, proliferation and differentiation have not yet been thoroughly investigated.

4.4.2. Coatings

Coating of microparticles with ECM proteins is the most common approach to increase cell attachment on microparticles. Many natural biomacromolecules have domains in their structure, which cells regognize as ligands that can specifically bind with integrins on the cell membranes. This specific recognization can result in accelerated cell attachment and spreading. (Hong et al. 2005) For instance, collagen, laminin, fibronectin, gelatin and Matrigel™ have been used for coating biostabile and biodegradable microparticles. Examples of coating experiments of microparticles with ECM proteins to increase cell attachment on microparticles are listed in Table 2.

Table 2. *Coating microparticles with ECM proteins.*

Material	***Coatings***	***Investigated cell type***	***Source***
Cellulose DE 53	Laminin, Matrigel™	hESCs	Oh et al. 2009; Chen et al. 2011
Cellulose DE 53	Fibronectin,Vitronectin	hESCs	Chen et al. 2011
Cytodex ™ 1	Laminin	hESCs	Lecina et al. 2010
Cytodex™ 3	Matrigel™	hESCs	Nie et al. 2009; Fernandes et al. 2009; Chen et al. 2011
Hyclone™	Matrigel™	hESCs	Lock et al. 2009
PLA	Collagen	Chondrocytes	Hong et al. 2005; Hong et al. 2008
PLA	Chitosan	Chondrocytes	Lao et al. 2008
PLGA	Fibronectin	Neural stem cells	Bible et al. 2009
PLGA	Fibronectin	MSCs	Bouffi et al. 2010
PLGA	Gelatin	ESCs	Carpenedo et al. 2010
PLGA	Laminin	P19 embyonic carcinoma cells	Newman et al. 2004
PLLA:PLGA blends	Laminin	ARPE-19	Thomson et al. 2010
Polyhydroxyethyl-methacrylate(PHEMA)	Laminin, Fibronectin	Hepatocytes	Denizli et al. 1995
TOSOH™ 65	Matrigel™, Laminin, Fibronectin,Vitronectin	hESCs	Chen et al. 2011

Several studies have reported improved cell survival in microparticle culture systems with ECM-coated microparticles. For example, Thomson et al. compared the attachment of ARPE-19 cells on uncoated and laminin-coated blended PLLA and PLGA microparticles. Interestingly, no significant difference in cell attachment between

laminin-coated and uncoated microparticles was detected by light microscopy. However, when cell viability was investigated with lactate dehydrogenase assay, a trend towards improved cell viability on laminin-coated particles was seen. (Thomson et al. 2010) In another study, Bible et al. reported improved attachment and more even distribution of neural stem cells on fibronectin-coated PLGA microparticles compared to uncoated particles (Bible et al. 2009). Chen et al. investigated several ECM proteins for improving attachment and viability of hESCs on multiple commercial microparticles. hESC growth was improved on microparticles coated with ECM proteins, and laminin and Matrigel™ proved to be essential for stable long-term propagation of hESCs on variety of microparticles. (Chen et al. 2011)

4.4.3. Charge and hydrophilicity

History of microparticles started with positively charged microparticles, but later it became evident that negatively charged materials as well as amphoteric materials such as proteins can also be used (Nedovic et al. 2004). The first microparticles used for cell culture purposes were hydrophilic ion-exchange chromatographic beads based on cross-linked dextran (van Wezel 1967). Biodegradable polymers, such as PLGA and PLAs, have been known to be materials of which surfaces are quite hydrophobic and have almost no reactive groups in their molecules (Takami et al. 2011). Therefore, efforts to increase the hydrophilicity and overall charge of microparticles are required.

Addition of charged groups such as DE, polyethylene glycol (PEG) and poly(L-lysine) have been shown to increase cell attachment and proliferation on microparticles. For instance, the effect of PLGA surface charge on chondrocyte attachment on microparticles has been investigated. Three different kinds of PLGA microparticles having hydrophobic, negatively charged surface and positively charged surfaces were compared with each other. The negative surface charge was achieved by preparing the particles from uncapped PLGA polymer whereas blending PLGA with PLGA-g-poly(L-lysine) graft polymer resulted in positively charged particle surfaces. The positively charged PLGA microparticles showed the highest cell attachment and proliferation compared to the hydrophobic and negatively charged particles. (Chun et al. 2004) PLA and PLGA microparticles have been modified with PEG as well. Introduction of PEG to the particle surface increased the Zeta potential of the particles. (Takami et al. 2011)

One approach to functionalize the surface of biomaterial with reactive groups is by means of plasma processing. Plasma is a complex mixture composed of free radicals, ions, electrons, atoms, and molecules sustained by an external energy source. (Chu et al. 2002) Plasma polymerization modifies the surface chemistry of the microparticles, increases hydrophilicity and provides sites for the covalent attachment of functional groups (Bible et al. 2009). For example, cell attachment on polystyrene microparticles can be significantly increased with alkylamine plasma treatment (Piskin 1992). Allylamine plasma polymerization of PLGA microparticles have resulted in improved coating efficacy and cell attachment of neural stem cells (Bible et al. 2009).

Alternative method to induce the hydrophilicity and change the charge of the particle surface is aminolysis. Surface aminolysis is performed to introduce reactive NH_2 groups, which are transferred into aldehyde groups by reaction with glutaraldehyde. These aldehyde groups can react with aminogroups of other molecules, such as collagen. Immobilization of collagen on PLA microparticles with surface aminolysis resulted in chondrocyte attachment and proliferation on particles. In addition, larger amounts of collagen were immobilized on microparticles with higher NH_2 content. (Hong et al. 2005)

Treating PLLA microparticles in aqueous solutions of sodium hydroxide (NaOH) have reported to results in formation of carboxyl groups to particle surface. These carboxyl groups increase the hydrophilicity of the particle surface, and allow the immobilization of peptides. PLLA microparticle surfaces have been immobilized with RGD peptide motif with NaOH-treatment. RGD modified particles enhanced adhesion and proliferation of chondrocytes in PLA microparticle cultures. (Chen et al. 2006)

4.4.4. Porosity and blend ratio

Solid, microporous and macroporous microparticles have been used in suspension cultures. Porous microparticles fabricated from biodegradable polymers show great potential as substrata for cell cultivation in tissue engineering (Shi et al. 2009). Macroporous microparticles have pores in the range between 10 to 400 μm, allowing the cells to grow inside the pores. Microporous particles have so small pore size that the cells cannot penetrate inside the particles forcing the cells to grow as a monolayer on the particle surface. (Castilho et al. 2008)

Porous PLA microparticles have been prepared with double emulsion solvent evaporation method. Good cell attachment and spread on these porous PLA particles was seen with human osteosarcoma cells. (Shi et al. 2009) In another study, porous PLGA microparticles resulted in improved preadipocyte cell survival compared to nonporous particles. (Chung et al. 2009) hESCs have been cultured on macroporous commercial Cytopore 2 and Cultispher^{TM} G microparticles as well. However, low cell yields were reported. The macroporous microparticles might provide a non-uniform exposure of cells to nutrients and growth factors, whereby cells inside the pores have less access to growth factors. In addition, Cultispher^{TM} G cultures resulted in a decrease of pluripotency. (Chen et al. 2011)

The composition of the microparticle material has an effect on the outperformance of particles in cell culture studies. The properties of microparticles composed of copolymers or polymer blends are affected by the blend ratio. Gabler et al. investigated the effect of blend ratio to the resulting PLGA particle size. Particles with low PLA content were smaller than particles obtained with the same parameter set with higher PLA ratio. In addition, lower PLA proportion led to faster surface erosion of the particles. Interestingly, particles with high PLA content showed best results in respect of chondrocyte adherency. (Gabler et al. 2007) Thomson et al. also studied the effect of blend ratio of PLGA to the resulting particle size with single emulsion based method.

Their results were consistent with Gabler et al. The blends rich in PLA produced larger particles and were superior for maintenance of the RPE cell viability as well. (Thomson et al. 2010)

4.5. Microparticles in cell culture and tissue engineering

Cell attachment to the microparticles depends on the chemical composition, surface topography, porosity and surface charge of the particles. Furthermore, the diameter of the particles and resulting surface affect the number of attached cells. The major advantage of microparticles is a relatively large surface area in a relatively small volume. For instance, 1 g of Cytodex^{TM} 1 microparticles has a surface area of 4400 cm^2, which corresponds to the surface are of fifty-eight 75 cm^2 culture flasks. Thus, the use of microparticles in cell and tissue culture is space saving, cost effective and less time and labor consuming than conventional 2D culture methods. In addition, serial samples can be taken from the cultures without disturbing the cell proliferation. Moreover, microparticle cell culture systems allow adjustment of culture conditions, such as controlling the composition of the culture media and environmental factors as shear force. Besides cell culture system, microparticles have been used in cell behaviour studies as well as cell delivery systems to regenerate tissue in trauma sites. (Martin et al. 2011)

4.5.1. Microparticles and primary cells

Several studies have proven that primary cells can be cultured efficiently on microparticles and in large scale in bioreactors. In particular bone and cartilage forming cells, keratinocytes, hepatocytes and cells for central nervous system have been investigated in microparticle culture systems. Furthermore, several microparticle-expanded primary cells have been used for transplantation purposes in animal studies. (Martin et al. 2011; Sun et al. 2011)

Microparticles have intrigued in bone graft engineering due to the rapid cell growth and expansion on particles. Furthermore, these microparticles can be incorporated into larger scaffolds with suitable mechanical stability for transplantation purposes. Interestingly, the shear stress in perfused cultures has been shown to have a positive effect on osteogenic cells. (Martin et al. 2011) Osteoblasts and other osteogenic progenitor cells have been shown to proliferate on variety of microcarriers, and have been used in both *in vitro* and *in vivo* investigations. (Qiu et al. 2000; Botchwey et al. 2003; Barrias et al. 2005) Also chondrocytes have been proven to retain their phenotype when cultured and propagated on microparticles. Moreover, chondro-progenitor cells on microparticles have been shown to produce tissue-like materials. Microparticles composed of materials such as PLA, PLLA and PLGA have been used for cultivation of chondrocytes either alone, or embedded inside a hydrogel. The embedded microparticles in hydrogels inhibited the uncontrollable movement of the particles,

whereas microparticles enhanced the mechanical strength of the hydrogel. (Martin et al. 2011; Hong et al. 2008)

Keratinocytes are traditionally cultured on a layer of lethally irradiated mouse 3T3 fibroblasts that support their growth. Avoiding the need for these feeder cells with culturing keratinocytes on microparticles would be advantageous for transplantation purposes. Voigt et al. showed that keratinocytes can proliferate on Cytodex™ microcarriers, and these cell and particle complexes could be cryopreserved. However, the Cytodex™ are not biodegradable and resulted in scar formation after implantation in a mouse model. (Voigt et al. 1999) Keratinocytes have also been cultured on biodegradable PLLA, PLGA and commercial Cultispher™ microparticles. These studies have shown that microparticle culture system supports the growth of keratinocytes without mouse fibroblasts. Interestingly, keratinocyte transplantation on microparticles has been studied in a clinical study in which autologous human keratinocytes were used to treat venous leg ulcers. Partial or full re-epithelialization was seen in all wounds treated with keratinocytes on Cultispher™ microparticles. (Liu et al. 2004; Martin et al. 2011)

In addition to osteoblasts, chondrocytes and keratinocytes, hepatocytes have also been vigorously studied in microparticle culture systems. The first reports of hepatocyte expansion were reported already in 1986, in which rat hepatocytes were cultured on Cytodex 3™ microparticles. The investigators reported prolonged hepatocyte viability and function *in vitro* as well as *in vivo*. (Demetriou et al. 1986) In addition to expansion of cells *in vitro* and transplantation studies in animal models, microparticles have been investigated for the use in bioartificial liver devices (BAL). BALs are bioreactors usually composed of a hollow fibre that supports circulating hepatocyte culture. Microparticles made of cellulose, chitosan, collagen, gelatin, plastic and PLGA have been studied as potential parts of these BAL systems. (Martin et al. 2011; Sun et al. 2011)

Ocular pigment epithelial cells have also been examined in microparticle suspension cultures. D'amico et al. were the first to report the culture of RPE cells on microparticles. They cultivated successfully mixed bovine retinal pigment epithelium-choroid cells on Superbeads. The RPE-choroid cells grew well on these microparticles, but contained considerably fewer pigment granules per cells. (D'amico et al. 1982) Kuriyama et al. cultivated human foetal RPE cells on Cytodex 3 microparticles in spinner flasks. They reported substantial increase in cell amount in a week, and also a bridging between the particles was seen, which according to the investigators are signs for tight cell adhesions. (Kuriyama et al. 1992) Thomson et al. studied blended PLLA and PLGA microparticles as suitable carriers for RPE cell transplantation. They seeded human RPE cell line ARPE-19 cells on particles coated with different ECM proteins. Particles rich in PLLA were superior for maintaining RPE cells viable. Also, laminin coating on the surface of the particles improved the RPE cell attachment. (Thomson et al. 2010) RPE cells have also been transplanted on microparticles. Oganesian et al. transplanted human foetal RPE cells on cross-linked fibrinogen microparticles to the

subretinal space of rabbits. A monolayer outgrowth of transplanted human foetal RPE cells from microparticles was seen as well as radiating hyperpigmentation around the transplanted microparticles. The cells survived in the subretinal space at least for a month, and a mild local inflammatory response was detected. (Oganesian et al. 1999) Human RPE cells have shown to produce levodopa. This property of RPE cells was utilized by Stover et al., who cultured RPE cells from human post mortem eyes on gelatin microcarriers and transplanted them to the striatum of six patients with advanced Parkinson's disease. The implanted microparticles and RPE cells were safe and well-tolerated, and improvements in the motor symptoms of the Parkinson's disease patients were seen. (Stover et al. 2005)

4.5.2. Microparticles and adult stem cells

The successful use of microparticles with primary cells has encouraged the use of these culture systems as an expansion tool of stem cells for clinical applications. MSCs are already in use in clinical studies, but technical problems such as long expansion periods have created a need for bioreactors that allow controlling and automation of the cell culture conditions. In fact, several studies have proven the ability of MSCs to attach, proliferate, and differentiate on microparticles in spinner cultures. For instance, Wu et al. were among the first to explore the performance of MSCs in microparticle cultures. They showed that the MSCs can be propagated over 10-fold in spinner cultures in 7 days, and the MSCs maintained their multipotency. (Wu et al. 2003) Especially Cultispher-S and Cultispher-G microparticles have been studied with MSCs as well as microparticles composed of hydroxyapatite. Besides MSCs, neural stem cells and pancreatic stem cells have been studied in microparticle suspension cultures. (Sun et al. 2011)

4.5.3. Microparticles and hESCs

Traditional culture methods of hESCs are impractical or unable to produce necessary scale-up of the cells for transplantation purposes. The usefulness of microparticles in cell culture has been proven with differentiated cells, but modifying microparticle culture system for hESCs is more challenging. (Martin et al. 2011) Fortunately, a few research groups have accepted the challenge, and developed microparticle culture systems for hESCs.

Phillips et al. investigated the attachment and proliferation of hESCs on Hillex II^{TM} microparticles in CM collected from hFFs. The cells were seeded on the microparticles either as single cells or as small aggregates. Phillips et al. reported that majority of the cells attached within 2 hours of cell seeding. The cultures were expanded on microparticles for 10 days. Single cell cultures resulted in a 3-fold expansion, whereas aggregates doubled the population. According to the investigators, the higher yield with single cells suspension might be due to a better coating by cells of a greater number of microparticles, therefore taking greater advantage of the surface

area. Furthermore, the single cell cultures were easier to passage and they persisted at least for 6 passages, whereas aggregated suffered from substantial cell loss and sustained only 3 passages. However, both single cells and cell aggregates expressed pluripotency markers throughout the culture time on microparticles. (Phillips et al. 2008)

Lock et al. studied the large scale production of hESCs on Matrigel™ coated Hyclone microparticles. As Phillips et al., Lock and his colleagues used CM, but their medium was collected from MEFs. After cell attachment on particles, the cells were transferred to spinner flasks, and the cultures were agitated. Different inoculum concentrations were studied and the high hESC seeding concentrations resulted in the formation of EB-like structures. Interestingly, the propagation of hESCs in bioreactor resulted in 34- to 45-fold increase in the number of hESCs during 8 days. Furthermore, the investigators stated that pluripotency markers TRA-1-81, Nanog, OCT 3/4 and SSEA4 did not deteriorate significantly during hESC propagation in microparticle cultures. (Lock et al. 2009)

Nie et al. had a different approach to culturing hESCs on microparticles. They modified the surfaces of Cytodex 3™ microparticles with either MEFs or Matrigel™. hESCs were effectively expanded in undifferentiated state on both Matrigel™-coated microparticles and microparticles seeded with a MEF monolayer. However, the expansion rate of hESCs on MEF-coated microparticles was slower than in conventional culture system, whereas the Matrigel™-coated microparticles were superior to the Matrigel™-coated tissue culture plates. Furthermore, Nie et al. showed in their study, that hESCs can be cryopreserved adherent on the microparticles with a higher recovery of undifferentiated cells than after cryopreservation of cells in suspension. (Nie et al. 2009)

Oh et al. tested hESC propagation in serum-free defined medias Stempro and mTeSR® 1. They cultured hESCs on Matrigel™ coated DE 53 cellulose microparticles. They showed that this type of culture system allows simple and routine passaging of hESCs without differentiation. The expression of plurimarkers such as OCT 3/4, SSEA4 and TRA-1-60 were similar to those of the control 2D culture. After passaging the hESCs on these microparticles, they became fully confluent on day 6. In uncoated DE 53 microparticles, the cells rapidly colonized the particles, but underwent rapid differentiation. Histological analysis showed that hESCs on Matrigel™ coated microparticles formed multilayers of cells, and all of the cells still expressed plurimarker TRA-1-60. The hESC culture on microparticles resulted in doubling the cell yields in 7 days compared to conventional 2D cultures due to the increased surface area available. Due to the promising results with hESC culture on microparticles, Oh et al. investigated the large scale production of hESCs in spinner flasks. This resulted in a superior density compared to static microparticle conditions and over 4-fold yields compared to 2D cultures. These hESCs were cultured on microparticles up to 7 weeks with continuous expression of plurimarkers. (Oh et al. 2009)

Fernandes et al. reported successful scale-up of hESCs on microparticles in feeder- and Matrigel™-free conditions in continuous agitation. Fernandes and colleagues studied Cytodex™ 3 microparticles in MEF CM. They cultured hESCs under continuous agitation for 15 days resulting in 2-fold yields compared to static 2D-cultures. These cells maintained stable karyotype after two weeks of cultivation. (Fernandes et al. 2009) Storm et al. focused on the initial seeding parameters of hESCs on Cultispher- S microparticles. To improve the cell survival in passaging, they used a p160-Rho-associated coiled kinase (ROCK) inhibitor Y-27632. With ROCK inhibitor, the cells were successfully passaged as aggregates. 3D cultures of hESCs in MEF CM maintained expression pluripotency markers, which in some cases were even higher than in conventional 2D cultures. (Storm et al. 2010)

4.6. Differentiation of hECSs on microparticles

Even though several studies on culturing hESCs on microparticles have been reported, only few research groups have attempted direct differentiation of hESCs towards mature cell types on microparticles. Lock et al. differentiated hESCs on collagen coated Hyclone™ microparticles towards definite endoderm with a medium containing activin A, Wnt3a, and low concentration of serum. More than 80% of the differentiated hESCs expressed definite endoderm markers, such as forkhead box protein A2 (FOXA2) and SOX17, whereas the expression of non-definite endoderm genes was minimal. (Lock et al. 2009) Lecina et al. investigated the effect of several microparticles on differentiation efficiency of hESCs towards cardiomyocytes. For example, DE 53, Cytodex™ 1 and 3, FACT and TOSOH-10 microparticles were used for studying the effects of type, size, shape and microparticle concentration on the differentiation efficacy. Lecina et al. reported that the smaller the beads, the more regular and smaller aggregates were formed, which enhanced the differentiation. The hydroxylated metacrylate TOSOH-10 microparticles with a diameter of 10 μm produced 80% of beating aggregates out of all aggregates, threefold cell expansion and yields of 20% of cardiomyocytes. (Lecina et al. 2010) Leung et al. focused on the influence of agitation on differentiation of hESCs in microparticle cultures. They claimed that the mechanical stress caused by agitation resulted in downregulation of OCT3/4, mAb 84, and TRA-1-60 plurimarkers. However, they concluded that the changes in the differentiation of the agitated cell line does not result directly from the shear stress caused by agitation, but rather by signaling effects that influence the cells to differentiate resulting in slower growth. (Leung et al. 2010) Also, differentiation towards ectoderm has been achieved on Hillex II™ and Cultispher™ particles. (Phillips et al. 2008)

EXPERIMENTAL PART

5. MATERIALS AND METHODS

The practical work in this thesis consisted of two fields, first being the preparation of biodegradable microparticles and the optimization of the analysis of prepared particles. The second part of the practical work included culturing and differentiation of hPSCs on self-prepared and commercial microcarriers. The course of the study is represented in Figure 11.

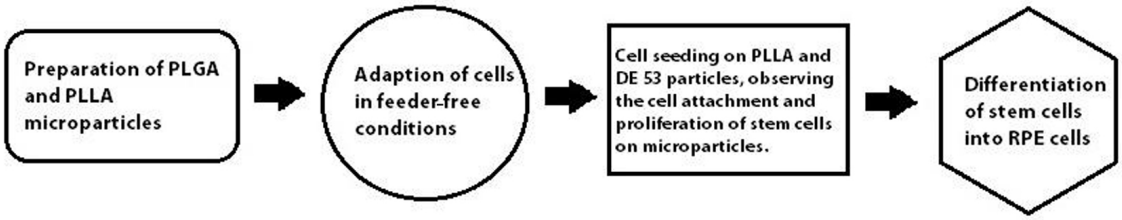

Figure 11. *The course of the practical work.*

5.1. Microparticles

5.1.1. Materials for particle preparation and commercial microcarrier

Biodegradable PLGA granules with the composition of 53/47 PLDLGA from Bio Invigor (Bio Invigor Corporation, Taipei, Taiwan) were used for microparticle preparation. The PLGA granules were a kind gift from our collaborator professor Loo. These copolymer granules had the inherent viscosity (i.v.) of 1.03 dl/g. In addition, Purasorb PL 24 PLLA granules with i.v. of 2.51 dl/g were purchased from Purac (Purac Biomaterials, Gorinchem, Netherlands). The PLLA had maximum of 0.01 % of residual solvent and 0.1 % of residual monomer. Both polymers were used in concentration of 0.04 g/ml. As comparison, commercial preswollen microgranular cellulose anion exchange diethylaminoethyl (DE) 53 microcarriers from Whatman (Whatman international Ltd, Kent, UK) were used. These microcarriers are positively charged due to the DE tertiary amino groups and possess a small ion exchange capacity of 2 milli-equivalents per gram of dry materials. DE 53 microparticles are cylindrical in shape and have the dimensions of 40 to 50 μm in diameter and 80 to 400 μm in length, and they are more commonly used in chromatography due to their anion exchange capacity (Figure 9).

5.1.2. Preparation of biodegradable microparticles

Biodegradable PLGA and PLLA microparticles were prepared according to the protocol of our collaborator Joachim Loo and his colleagues with slight modifications. The preparation protocol was a simple and versatile one-step solvent evaporation technique, which was not only quite fast to execute but also economical since complex and expensive fabrication equipment were not needed. (Loo et al. 2010) Figure 12 illustrates the fabrication protocol of microparticles by a single emulsion process.

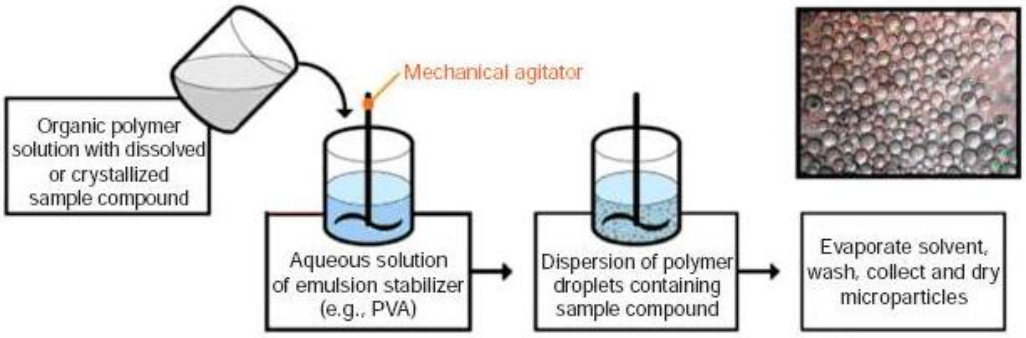

Figure 12. *A Schematic picture about microparticle preparation by a single emulsion process. (Bible et al. 2009)*

First, a polymer solution was prepared. 200 mg of desired polymer was added into 5 ml of DCM (Sigma-Aldrich, St. Louis, MO, USA) and stirred with a magnetic stirrer for 2 h at room temperature (RT) until the polymer granules were fully dissolved into the solvent. Simultaneously, an aqueous solution of emulsion stabilizer was prepared. In this study, 1% PVA solution was used. 500 mg of PVA powder (Bio Invigor) was dissolved in 50 ml of deionized water and stirred with a magnetic stirrer for 2 h at RT. The prepared PVA solution was sterile filtered with a 0.2 µm filter and a syringe.

5 ml of 1 % PVA solution was added into a glass container, after which 1 ml of prepared polymer solution was carefully added on top. Two separate immiscible phases were detected. A magnetic stirrer was dropped into the container very gently and the container was sealed with either two layers of parafilm or a cork depending on the used vessel. Few holes were made into the parafilm so that the fumes formed in the preparation process were able to exit the container without tearing the whole parafilm layer. In order to optimize the preparation protocol for the equipment and reagents found in our laboratory, several glass containers with different shapes and sizes were tested in addition to few different magnetic stirrer sticks with distinct dimensions.

The stirring speed has a huge impact on the size and density of the formed particles. The desired particle size in this experiment was approximately from 100 to 150 µm. To achieve particles of this size, particle preparation paramaters such as stirring rate were optimized. The mix of solutions was either stirred with constant speed

for 2 h or quickly swung by hand and left still for the rest of the 2 h. In this study, stirring speeds of 0 rpm, 120 rpm and 500 rpm were tested. After stirring, the parafilm was replaced by a sterile gas mask, and the container was left in fume hood over night so that the DCM solvent was fully evaporated from the solution. Next, the particles were transferred into 2 ml eppendorfs, approximately 50 mg of microparticles per tube, and washed twice with sterile water. In between the washes, the beads were centrifuged down with the speed of 6500 rpm for 5 minutes. After the final centrifugation, the pellet was resuspensed with 30 µl of sterile water, and the eppendorfs were left open in laminar hood for 3 to 4 days to dry. Steps of particle preparation are illustrated in Figure 13.

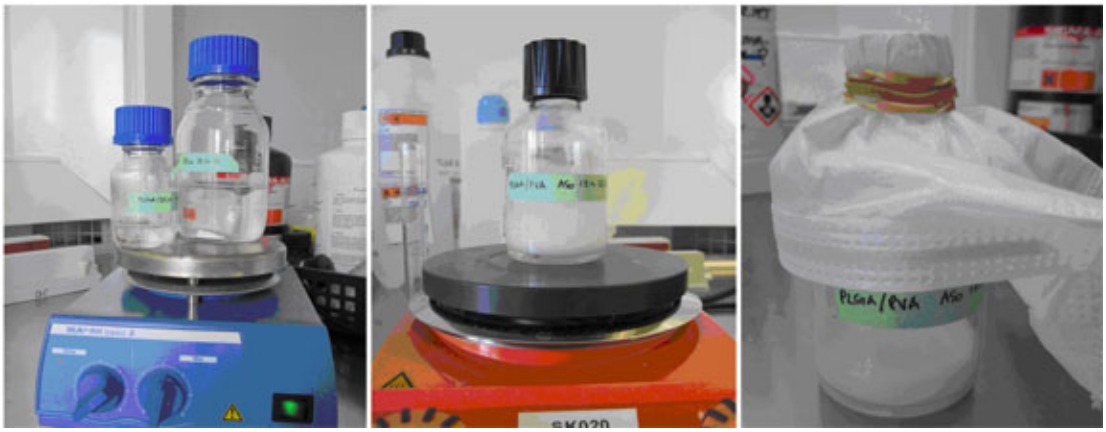

Figure 13. *Pictures about particle preparation. On the left, preparation of polymer and emulsion stabilizer solutions. In the middle, mixing of the solutions with a magnetic stirrer. On the right, the solvent is evaporated over night by covering the container with a sterile gas mask.*

5.1.3. Coating of microparticles

In order to improve the cell attachment on microparticles, different coatings were tested. Prepared PLLA beads and commercial DE53 beads were coated with few ECM proteins. For this study, BD Matrigel™ (BD Biosciences, Franklin Lakes, NJ, USA) and mouse laminin (Sigma-Aldrich) were chosen. One tube of each particle type (á 50 mg of particles) was resuspended into Dulbecco's Phosphate Buffered Saline (DPBS) and divided into three 5 ml sterile tubes. The beads were allowed to settle down to the bottom of the tube and excess DPBS was removed. Three different solutions for each particle type were prepared; BD Matrigel™ dilution (0.33 mg/ml), mouse laminin solution (20 µg/ml) and DPBS as a control. The tubes were sealed with caps, and parafilm was applied on top after which the beads were coated overnight in a Roto-Shake shaker at 4 ºC. For the coating, the tubes were attached to a shaker so that the solution and microparticles inside the tubes moved from one end of the tube into another with a very slow speed (Figure 14).

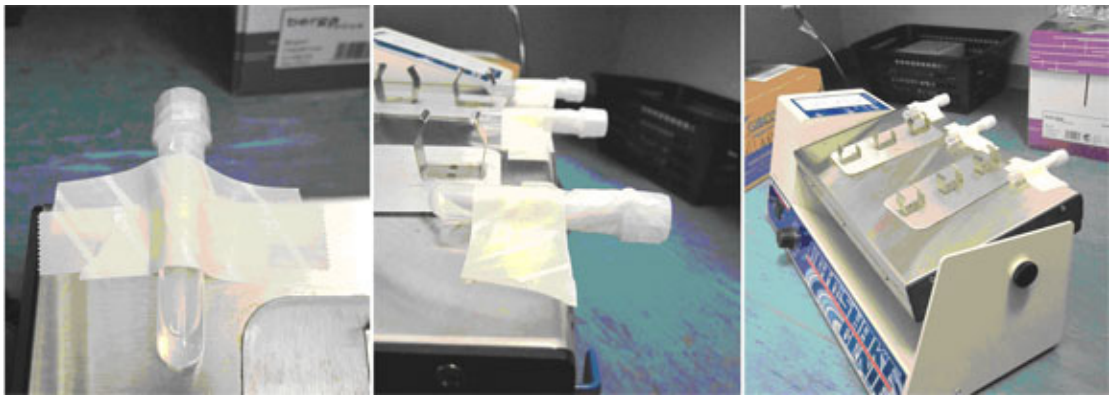

Figure 14. *Coating microparticles on a Roto-Shake shaker.*

5.1.4. Autofluorescence and background

Immunofluorescence and imaging was a major analysis tool in this study. In preliminary studies conducted in the ophthalmology group of BioMediTech, a strong background with common immunoreagents was detected with the PLGA microparticles. Therefore, before cell seeding on the microparticles, the intensity of autofluorescence and background with 4',6-diamino-2-phenylindole (DAPI) were investigated with different wavelengths.

First, autofluorescence of self-prepared and commercial particles was tested. Dried self-prepared particles and commercial DE 53 were resuspended into sterile water and small amounts of particles were added onto microscope coverslips and excess water was removed. A drop of Vecta Mount^{TM} without DAPI (Laboratories, Inc., Burlingame, USA) was applied on top of the particles, and the samples were covered with cover glasses. Excitation wavelengths of 320 nm, 488 nm and 568 nm were inspected, and images were taken with Olympus IX 51 Fluorescence microscope (Olympus Europa Holding GMBH, Hamburg, Germany).

In preliminary studies DAPI caused significant background that disturbed the analysis of cell attachment and proliferation on microparticles. Therefore the background caused by DAPI with different dilutions for all the particles under inspection was examined. A dilution series of Vectashield Mount^{TM} with DAPI (Vector Laboratories, Inc., Burlingame, USA) was prepared according to the Table 3. Dry particles were resuspended into sterile water and small amounts of particles were transferred to the bottoms of 48-well plate wells. A drop of undiluted mount with DAPI or diluted mount with DAPI was applied on top of the samples. The wells were covered with cover glasses, and the background with DAPI was analyzed and imaged with Olympus IX 51 Fluorescence microscope.

Table 3. *DAPI dilution series for testing background caused by DAPI.*

	1:10	**1:100**	**1:1000**
DAPI Dilution series	30 µl of vectashield mount with DAPI	30 µl of 1:10 DAPI dilution	30 µl of 1:100 DAPI dilution
	270 µl of PBS	270 µl of PBS	270 µl of PBS

5.1.5. Analysis of particle preparation and coating procedures

The size and shape of self-prepared microparticles were analysed by microscopy with Olympus IX 51 fluorescence microscope. Dried particles were suspended into DBPS, and transferred to a well of a 48-well plate. Both bright field and fluorescence images were taken.

The success of coating procedure of PLLA and DE 53 particles was verified by indirect immunofluorescence staining. In indirect immunofluorescence, the sample is first treated with a primary antibody that binds specifically into the investigated molecule. Next, the sample is incubated in secondary antibody dilution that binds the primary antibodies or their fragments. These secondary antibodies are usually labelled with fluorescent molecules which allow the visualization of the inspected molecules with fluorescence microscope. In this study, the following protocol was used for immunofluorescence stainings. Secondary antibody without primary antibody was used as a control. The background of PLLA and DE 53 microparticles with secondary antibody was investigated from the same control samples.

The immunofluorescence protocol for microparticles was carried out in 1.5 ml eppendorf tubes. The particles were washed three times with DPBS to remove any remainings of the coating dilutions. To block unspecific protein binding, the samples were treated with 3% bovine serum albumin in DPBS (BSA-DPBS) for 1 h at RT. The primary antibody used for detecting the presence of coating was anti-laminin (Abcam, rabbit IgG, 1:100). The primary antibody was diluted into 0.5% BSA-DPBS, and the samples were incubated for 1 h at RT. After the incubation, samples were washed three times with DPBS. Secondary antibody dilution was also prepared into 0.5 % BSA-PBS. In this study, secondary antibody Alexa fluor 568 anti-rabbit IgG (Molecular Probes, Invitrogen) was used with dilution ratio of 1:1500. The samples were incubated in secondary antibody dilution for 1 h at RT, after which the samples were washed again three times with DPBS and once with milli-Q-water. The stained microparticles were transferred onto cover glass and a drop of Vecta Mount^{TM} without DAPI was applied on top. The sample was covered with another cover glass. Imaging and visualization of the success of the coating was carried out with Olympus IX 51 fluorescence microscope.

5.2. Cell culture methods

5.2.1. Cells and cell culture material

hESC lines Regea 06/040 (46, XX) and Regea 08/023 (46, XY) established in former Regea Institute for Regenerative Medicine (currently known as BioMediTech) in University of Tampere were used. Regea has an approval of the National Authority for Medicolegal Affairs Finland to study human embryos (Dnro 1426/32/300/05). In addition, the Ethics Committee of Pirkanmaa Hospital District has given a supportive statement for Regea for the derivation, characterization and differentiation of hESC lines (Skottman/R05116). These hESC lines are derived from early stage embryos that could not be used in infertility treatments. The donors of the embryos signed an informed consent form and they did not receive any payment. The hiPSC line FiPS 5-7 derived by Professor Otonkoski's group at the University of Helsinki was also investigated. This hiPSC line was generated from human fibroblasts utilising four transcription factors: OCT 3/4, SOX2, nanog, and LIN28. Both hESCs and hiPSCs were cultured on top of irradiated hFFs. The cell lines were maintained in serum-free hESC medium at + 37 ^{o}C in 5 % CO_2. The cells were mechanically passaged on to new hFFs every 6 to 7 days. (Skottman 2010)

5.2.2. Feeder-free culturing

Before seeding cells on microcarriers, the hESCs and hiPSCs were adapted in feeder-free conditions for 3 to 8 passages. The hESC and hiPSC colonies on feeder cells were cut into small pieces with a scalpel and transferred onto growth factor reduced Matrigel^{TM} (BD Biosciences) coated 6-well plates (Corning Incorporated, MA, USA). The cut pieces were detached carefully as sheet-like pieces. Approximately 150 colonies were cut and transferred onto 2 to 3 wells. Serum-free and defined mTeSR$^{®}$1 media (Stemcell Technologies, Grenoble, France) for the feeder-independent maintenance and expansion for hESCs in undifferentiated state was used, and the medium was prepared according to the manufacturer's instructions with the modification of adding antibiotics (0.5% penicillin/streptomycin, Lonza Group Ltd). The cells were fed with 2 ml of fresh medium five days a week and double fed with 4 ml of fresh medium once a week. Prior to feeding, the state of the hESC and hiPSC colonies was checked with a phase contrast microscope (Nikon Eclipse TE-2000s, Nikon Instruments) or a stereomicroscope (Nikon SMZ800, Nikon Instruments).

The hESC and hiPSC colonies required to be passaged onto new 6- or 12-well plates coated with Matrigel^{TM} every 4 to 6 days. For coating, the Matrigel^{TM} was thawed on ice. Furthermore, all the pipette tips, well plates and tubes used for the coating procedure were cooled in +4 ºC. Matrigel^{TM} was diluted into ice cold DMEM/F-12 (Gibco, Invitrogen, NY, USA) to final concentration of 0.083 mg/ml. 1 ml of

Matrigel™ dilution was added into a well of a 6-well plate and 0.5 ml into a 12-well plate well. Well-plates were coated with Matrigel™ overnight at +4 ºC.

The colonies in feeder-free conditions were ready to be passaged, when the centers of the colonies became dense and phase-bright compared to their edges, and the adjacent colonies started to merge. Prior to passaging, differentiated colonies and regions were removed with a 1000 µl pipette tip or a needle. The removed pieces and medium was aspirated and the wells were rinsed with 2 ml of DMEM/F-12. 1 ml of dispase (Gibco, Invitrogen) with the concentration of 1.7 U/ml was added into each well. The plate was placed at +37 ºC for 7 minutes or until the edges of the colonies started to slightly fold back from the bottom of the well, but the colonies still remained attached to the plate. The dispase was removed and the wells were gently rinsed with DMEM/F-12 to dilute and wash away any remaining enzyme. 2 ml of DMEM/F-12 was added per well, and the colonies were scraped off carefully with a scalpel or a 5 ml pipette tip so that the colonies stayed as sheets. The detached colonies were transferred to a 15 ml falcon tube and the wells were rinsed with 2 ml of DMEM/F-12 to remove the remaining colonies. The cell aggregates were centrifuged with Heraus® Biofuge® Primo (Kendro, Germany) at 1400 rpm for 5 minutes. The supernatant was removed and the cell pellet was carefully resuspended into 1 ml of mTeSR™ 1 media. The hESC and hiPSC aggregates were plated evenly to a new well-plate coated with Matrigel™ in diverse ratios depending on the cell density.

5.2.3. Culturing cells on microcarriers

Before inoculation of the cells on particles, the commercial DE 53 particles were autoclaved, and adapted in DPBS overnight at +4°C, whereas the self-prepared PLLA particles were surface sterilized with UV-light for 25 min. After sterilization and adaptation procedures, the particles were coated in Matrigel™ over night as described in Chapter 5.1.3. The coated beads were washed twice with DMEM/F-12 and once with mTeSR® 1 medium to remove the excess Matrigel™ dilution. The hESC and hiPSC cultures that were seeded on microparticles had been adapted in feeder-free conditions 3 to 8 passages. Differentiated areas were removed from cultures, and the wells were rinsed with DMEM/F-12 to remove the remains of differentiated cells. Next, the cell colonies were treated with 1 ml of prewarmed Tryple (Gibco, Invitrogen) for 5 min in +37 °C, after which most of the cells had detached as small aggregates. 4 ml of warmed DMEM/F-12 was added to inactivate the enzyme, and the cell aggregate suspension was transferred into 15 ml falcon tubes and centrifuged with Heraus® Biofuge® Primo 1300 rpm for 5 min. The supernatant was removed and the cell pellet was resuspended into 500 µl or 1000 µl of mTeSR®1 media. Cells were calculated with Neubauer Improved hemocytometer. A small amount of coated beads was transferred to the bottom of a 24- or 96-low cell bind well plates (Corning Incorporated). Appropriate amount of cells was added on to the particles according to the Table 4.

Table 4. *Used cell to particle ratios and well-plate sizes in cell seeding on microparticles.*

Particle type	*Well-plate*	*m (particles)*	*Cell line*	*Amount of cells seeded*	*Cell to particle ratio*	*Agitation*
DE 53	24	25 mg	hESC 06/040	500 000	20 000 cells/mg	No
DE 53	24	25 mg	hiPSC 5-7	1 850 000	74 000 cells/mg	No
DE 53	24	25 mg	hiPSC 5-7	1 850 000	74 000 cells/mg	Yes
DE 53	96	10 mg	hiPSC 5-7	1 000 000	100 000 cells/ mg	No
DE 53	96	10 mg	hiPSC 5-7	1 000 000	100 000 cells/ mg	Yes
DE 53	96	15 mg	hiPSC 5-7	350 000	23 300 cells/mg	No
DE 53	96	10 mg	hiPSC 5-7	1 000 000	100 000 cells/ mg	No
DE 53	96	10 mg	hESC 08/023	1 000 000	100 000 cells/ mg	No
PLLA 120 rpm	96	15 mg	hiPSC 5-7	350 000	23 300 cells/mg	No
PLLA 120 rpm	96	20 mg	hiPSC 5-7	1 000 000	50 000 cells/ mg	No
PLLA 120 rpm	96	15 mg	hESC 06/040	139 000	9300 cells/mg	No
PLLA 0 rpm	96	20 mg	hiPSC 5-7	1 000 000	50 000 cells/ mg	No
PLLA 0 rpm	96	20 mg	hESC 08/023	1 000 000	50 000 cells/ mg	No

5.2.4. Effect of agitation on cell attachment and proliferation

The effect of agitation on cell attachment and proliferation in microparticle suspension cultures was investigated for DE 53 particles. During the cell seeding on particles, two parallel wells with similar cell to particle ratio were prepared. The other was agitated during day 0 and the other one was left still. The agitation was conducted by pipetting media up and down with a 1000 µl pipette so that the cells and beads on the bottom of the well were mixed. The agitation was repeated once every hour for 8 hours. In between the agitation, the cells and beads were allowed to settle down and the cells to attach while in contact with a microparticle surface.

5.2.5. Differentiation of hPSCs into RPE cells on microparticles

For the differentiation experiment, Regea 08/023 and FiPS 5-7 were seeded on DE 53 microparticles in a cell to particle ratio of 100 000 cells/mg in 96-well plate wells. The cell seeding on microparticles was conducted as decribed previously in Chapter 5.2.3. On day 2 after inoculation of cells, the mTeSR®1 medium for maintaining and expanding hPSCs was replaced with RPE differentiation medium (DM-). This RPE DM- medium consisted of Knockout™ D-MEM (Gibco, Invitrogen) supplemented with 15% Knockout™ Serum Replacement (Gibco, Invitrogen), 2 mM GlutaMax™ -1 Supplement (Gibco, Invitrogen), 1% MEM Non Essential Amino Acids (Lonza), 0.5% Penicillin/Streptomycin (Lonza) and 0.1 mM 2-mercaptoethanol (Gibco, Invitrogen). Fresh DM- medium was changed three times a week. After two weeks of culturing cells

in DM- on 96-well plates, the cells on beads were transferred onto low cell bind 24-well plates so that the volume of the medium could be increased.

5.3. Analysis of cell culture

5.3.1. Feeder-free culturing

The well-being of the hPSC lines in feeder-free cultures were analysed by phase contrast microscopy. The cells were followed and imaged daily. In addition, the pluripotency of Regea 08/023 and FiPS 5-7 was confirmed after 8 passages in feeder-free cultures with indirect immunofluorescence staining.

Briefly, the cells were fixed with 4% paraformaldehyde (PFA) for 10 minutes at RT, and washed afterwards three times with DPBS. Next, the cells were permeabilized with 0.1% Triton® X-100 (4-(1,1,3,3-tetramethylbutyl)phenyl-polyethylene glycol) (Sigma-Aldrich). The cells were permeabilized for 10 minutes at RT, and washed three times with DPBS. Then, to block any unspecific protein binding, the cells were incubated in 3% BSA-DPBS for 1 h at RT. Antibodies against plurimarkers OCT 3/4 and Nanog were used (Table 4). Primary antibody dilutions were prepared into 0.5% BSA-DPBS, and the samples were incubated for 1 h at RT. After primary antibody incubation, the samples were washed thoroughly three times with DPBS. As primary antibody dilutions, also secondary antibody dilutions were prepared into 0.5% BSA-DPBS. The samples were incubated in the secondary antibody dilution for 1 h at RT, after which they were washed again three times with DPBS. A drop of Vectashield Mount™ with DAPI was applied on top of the sample, and the sample was covered with a cover glass. Samples were stored at +4º C. The imaging was conducted with Olympus IX 51 Fluorescence microscope.

5.3.2. Cell attachment and proliferation on microparticles

Cell attachment to the microparticle surface and the cell proliferation was analysed with indirect immunofluorescence and phalloidin stainings on day 3 and on day 6 or 7 after cell seeding on particles. Small samples were taken from each well of microparticle suspension cultures. For DE 53, the sample size was approximately 5 mg, and for PLLA particles 10 to 15 mg. A similar immunofluorescence protocol was followed as described for the analysis of the pluripotency of the cells in feeder-free cultures. The staining protocol for microparticles was carried out in a 1.5 ml eppendorf tubes. The microparticles with cells on surface were as suspension in the culture medium, not attached to any surface. Therefore, after adding reagent solutions, the particles were allowed to settle down to the bottom of the tube and the supernatant was removed carefully with a pipette.

If phalloidin staining was done simultaneously with indirect immunofluorescence protocol, phalloidin was added to the secondary antibody dilution

mix. First, the solvent in phalloidin was evaporated in fume hood, and phalloidin was redissolved into the 0.5% BSA-PBS solution. The used primary and secondary antibodies in addition to phalloidin are listed in Table 5 with dilution ratios. DE 53 beads were transferred onto a superfrost microscope slide. A drop of Vectashield Hard Mount™ mounting media with DAPI (Vector Laboratories, Inc., Burlingame, USA) was applied on top of the sample and the sample was covered with a cover glass. The DE 53 samples were stored at +4 °C. Samples were imaged with Olympus BX 60 Fluorescence microscope. Due to the quite big particle size of investigated PLLA beads, they were hard to place between two cover glasses without the mounting media running away from the sample. Therefore, a PDMS structure was attached on a superfrost microscope slide. The PDMS structure had a hole in the middle, where the sample was added. A drop of Vectashield mounting media with DAPI was applied on top of the beads, and the samples were imaged immediately after the staining protocol. PLLA samples were visualised and imaged with Olympus IX 51 Fluorescence microscope.

Table 5. *The primary and secondary antibodies and other stainings used for immunofluorescence analysis of the cell culture.*

Stainings		***Manufacturer***	***Dilution ratio***	***Origin***	***Binds to***
Primary Antibodies	OCT 3/4	R & D Systems	1:400	Goat	A pluripotency marker, nuclear
	Nanog	R & D Systems	1:200	Goat	A pluripotency marker, nuclear
	Ki 67	Chemicon	1:500	Rabbit	A nuclear protein associated in cell proliferation
Secondary Antibodies	Alexa fluor 488 anti-goat Ig G	Molecular Probes, Invitrogen	1:1500	Donkey	A primary antibody originated from goat
	Alexa fluor 488 anti-rabbit Ig G	Molecular Probes, Invitrogen	1:1500	Donkey	A primary antibody originated from rabbit
Other stainings	Phalloidin	Sigma	1:200		Actin filaments

In case of conducting only a phalloidin staining without primary antibodies for the sample, a following protocol was performed. First, the appropriate amount of phalloidin was taken into an eppendorf, and dried in a fume hood for 1 to 2 hours. The samples were washed twice with DPBS and fixed in 4% PFA for 10 min at RT. After fixation, the samples were washed thoroughly three times with DBPS. The washes were followed by a permeabilization in 0.1% Triton® X-100 for 10 min at RT. The phalloidin was diluted into DPBS in ratio of 1:10. The samples were incubated in the diluted phalloidin for 10 min at RT and washed again three times with DPBS. Then the particles were transferred onto a microscope slide and mounted with Vectashield Mount™ with DAPI. Another cover glass was replaced on top of the sample, and the samples were stored at -20 °C.

5.3.3. Differentiation of hPSCs into RPE cells on microcarriers

The appearance of pigmentation was inspected with light microscope in pursuance of feedings during the whole culture time in the RPE differentiation medium. The maturation of cells was investigated more precisely with gene expression analysis by reverse transcriptase polymerase chain reaction (RT-PCR). RT-PCR is a qualitative analysis method, which reveals if certain genes are expressed in cells under inspection, but it does not give information about the quantities of gene expression levels. In RT-PCR, first RNA is isolated from the cells, which is followed by a complementary DNA (cDNA) synthesis. Finally, the cDNA is amplified using traditional PCR, and the gene expression of investigated genes can be visualized using agarose gel electrophoresis.

After 35 days in DM- RPE differentiation media, samples from both cell lines, 08/023 and FiPS 5-7, on DE53 were taken. The culture medium was removed, and the samples were washed with PBS. The cells were detached from the beads with Tryple. The detached cells were separated from the particles by filtration through a 40 µm cell strainer after which the samples were washed with PBS, and lysed into RA 1 lysis buffer with Reducing Agent tris(2-carboxyethyl)phosphine (TCEP). The lysis buffer inactivates RNases, therefore preventing the degradation of RNA during RNA isolation protocol. The samples were vortexed twice for 5 seconds, and stored at -70ºC to wait for further treatments. The RNA isolation was executed with NucleoSpin® RNA Xs kit (Macherey-Nagel, Düren, Germany). In Nucleospin® RNA XS kit the RNA is bound to a silica membrane in a column. The lysis buffer used for inactivating the RNase creates appropriate binding conditions which favor the adsorption of RNA to a silica membrane. However, contaminating DNA also binds to the silica membrane. This DNA is removed by a rDNase solution, which is applied directly on the membrane. Washing steps with two different buffers purify the sample from salts, metabolites and macromolecular cellular components. Pure RNA can be eluted from the silica membrane under low ionic strength conditions with RNase-free water. The amount of extracted RNA was measured with NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

The isolated RNA was synthesized into cDNA. Mature hESC 08/023-derived RPE cells (d196) and pluripotent hiPSC 5-7 (d0) were used as reference. The cDNA synthesis into RNA is based on the action of a reverse transcriptase enzyme, which translates the RNA into complementary cDNA. The cDNA synthesis was conducted with High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Briefly, a reagent solution containing the reverse transcriptase enzyme was prepared according to the Table 6.

Table 6. *The reagent solution of high-Capacity cDNA Reverse Transcription Kit for cDNA synthesis.*

Reagents	***Volumes used per one sample***
Sterile water	3.2 µl
10 x RT buffer	2 µl
25 x dNTP Mix	0.8 µl
10 x Rt random primers	2 µl
Multiscribe Reverse Transcriptase	1 µl
Rnase inhibitor dilution (10U/ml)	1 µl
Total	10 µl

In total 40 ng of isolated RNA was needed for cDNA synthesis per sample. Appropriate amount of sample was dilutes into sterile water so that the volume of dilution reached 10 µl. 10 µl of the prepared reagent solution was combined with 10 µl of the diluted RNA in a reaction tube, thus the total volume of the sample in the tube reached 20 µl. The cDNA synthesis was run with a cycle of 10 min at 25 ºC, 120 min at 37 ºC and 5 min at 85 ºC.

After synthesizing the samples into cDNA, the cDNA was multiplied in PCR reaction. Four genes were investigated; the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), the pluripotency marker *OCT 3/4*, and RPE specific markers *BEST* and *MITF*. *GAPDH* housekeeping gene was used as an endogenous control. The genes, their annealing temperatures, primers and product sizes are listed on Table 7.

Table 7. *The investigated genes in RT-PCR with appropriate primes, annealing temperatures and product sizes.*

Gene	***$T_{annealing}$ ^oC***	***Primers***	***5' > 3'***	***Size of the product***
GAPDH	55	GAPDH GAPDH	GTTCGACAGTCAGCCGCATC GGAATTTGCCATGGGTGGA	229 bp
OCT 3/4	62	OCT3/4-F OCT3/4-R	CGTGAAGCTGGAGAAGGAGAAGCTG AAGGGCCGCAGCTTACACATGTTC	245 bp
BEST	55	Bestrophin-F2 Bestrophin-R2	GAATTTGCAGGTGTCCCTGT ATCCTCCTCGTCCTCCTGAT	214 bp
MITF	52	Mitf-F Mitf-R	AAG TCC TGA GCT TGC CAT GT GGC AGA CCT TGG TTT CCA TA	352 bp

A reagent mix was prepared for each gene. Also controls without the reverse transcriptase enzyme were prepared (-RT controls). The reagent solutions for RT-PCR are shown in Table 8. 24 µl of the reaction solution was combined with 1 µl of the cDNA sample in PCR tubes. RT-PCR was started with a hot start, in which the samples were treated 3 min at 95 ºC. The rest of the program run consisted of 38 cycles of 30 s at 95 ºC, 30 s at the annealing temperature of the gene, and 1 min at 72 ºC. In this study

a temperature gradient was used from 51 ºC to 62.5 ºC so that the investigated genes could be studied in the same PCR synthesis.

Table 8. *Reagent solutions for each gene and –RT controls per sample.*

Reagent	***Sample***	***-RT control***
H_2O	16.6 µl	16.725 µl
10x buffer -Mg^{2+} and + KCl	2.5 µl	2.5 µl
MgCl 25 mM	1.5 µl	1.5 µl
dNTP 2 mM	1.25 µl	1.25 µl
primer-F 5 µM	1.0 µl	1.0 µl
primer-R 5 µM	1.0 µl	1.0 µl
Taq polymerase	0.125 µl	-

The expression of investigated genes was visualized with agarose gel electrophoresis. 2.0% agarose gel with a volume of 100 ml was prepared. The samples were loaded with 5 µl of 6x loading dye to visualise the loading and migration during the electrophoresis, and 20 µl of this sample solution was added to the gel. A generuler dilution with DNA ladder was used as a marker for product size in both ends of the gel. The gel was driven with 90 V for 50 minutes, which was followed by imaging the gel under UV-light for visualisation of the gene expression bands.

6. RESULTS

6.1. Particle preparation

In this study, biodegradable PLGA and PLLA microparticles were successfully prepared by a single emulsion process. Self-prepared particles with different average sizes and size distributions were manufactured by changing parameters such as stirring speed, size and shape of the vessel, and the size and presence of the magnetic stir bar. An optimal particle size for RPE applications is approximately 100 to 150 µm in diameter. For PLGA, particles of this size were manufactured without stirring in a 100 ml glass decanter. In contrast, for PLLA to achieve particles with similar size distribution, stirring with a magnetic stir bar with dimensions of 0.7 cm in width and 2 cm in length was required. Prepared PLLA particles are illustrated in Figure 15. Prepared particles and their average size and size distribution in addition to used parameters are listed in Table 9.

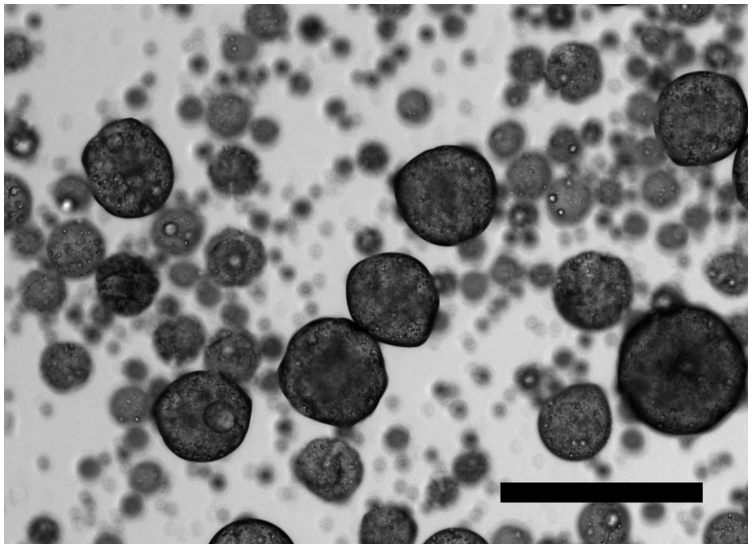

Figure 15. *Self-prepared PLLA particles with average size of 150 µm (scale bar 200 µm).*

Table 9. *Prepared biodegradable PLGA and PLLA microparticles by single oil-in-water emulsion process.*

Material	***Stirring speed***	***Stirring time***	***Magnetic stir bar***	***Vessel***	***Size distribution of prepared beads***	***Average size***	***Shape of the particles***
PLGA	0 rpm	-	-	100 ml glass decanter	10-150 µm	100 µm	Spherical, uniform in shape and size. Granules inside the beads. Particles were light in bright field images.
PLGA	0 rpm	-	-	25 ml glass erlenmeyer	only few smaller than 100 µm	100 to 150 µm	Elongated, spherical and balloon shaped. Particles were light in bright field images.
PLGA	120 rpm	2 h	3 cm x 1 cm	50 ml glass bottle	From nanoscale up to 100 - 200 µm	10 µm	Spherical
PLGA	120 rpm	2h	2 cm x 0.7 cm	100 ml glass decanter	10 to 200 µm lots of extremely small beads	Most of particles smaller than 20 µm	Spherical, wide size distribution. Particles were dark and grey in bright field images.
PLGA	500 rpm	o/n (20 h)	3 cm x 1 cm	75 ml glass bottle	From nanoscale up to 10 µm	Nanoscale	Mainly spherical and clear lined. Few donut-shaped and irregular particles.
PLLA	0 rpm	-	-	100 ml glass decanter	100 to 300 µm	150 to 200 µm	Spherical, uniform in shape and size. Small bubbles or granules inside the particles.
PLLA	0 rpm	-	-	100 ml glass decanter	Quite narrow size distribution	200 to 300 µm	Spherical and quite uniform in size
PLLA	120 rpm	2 h	2 cm x 0.7 cm	50 ml glass bottle	Smallest 10 to 50 µm, bigger ones 100 to 200 µm	50 to 100 µm	Partly irregular shape, elongated and balloon shaped particles detected
PLLA	120 rpm	2 h	2 cm x 0.7 cm	100 ml glass decanter	10 to 300 µm	100 to 150 µm	Spherical. Granules or air bubbles inside the beads
PLLA	120 rpm	2 h	2 cm x 0.7 cm	100 ml glass decanter	From smaller than 10 µm to 300-400 µm	150 µm	Spherical
PLLA	150 rpm	2 h	2 cm x 0.7 cm	100 ml glass decanter	From smaller than 10 µm to 300-400 µm	100 to 150 µm	Spherical

In these experiments, the 100 ml glass decanter vessel and a magnetic stir bar of 0.7 cm in width and 2 cm in length provided the most favourable circumstances for particle preparation. The bottom of the 100 ml glass decanter dish was quite even, which allowed the magnetic stirrer to whirl evenly. Also, a magnetic stir bar of this size was large enough to produce strong and even whirl, but still small enough to fit into the container.

Stirring speed of the polymer/PVA emulsion proved to have a major impact on the resulting particle size. The faster and stronger the stirring was, the smaller and denser particles were formed. In this study, a few stirring speeds were examined; 0 rpm, 120 rpm, 150 rpm and 500 rpm. With a stirring speed of 500 rpm, the prepared PLGA particles were all too small, almost nanoscale. For PLGA, the stirring speed of 120 rpm resulted also in too small average size with majority of the particles having a diameter around 10 µm. Suprisingly, with PLGA the protocol without stirring resulted in beautiful particles with quite even size distribution and average size of the particles approximately 100 µm. However, these stirring speeds did not have the same effect on prepared PLLA particle size. In general, the same stirring speeds produced larger PLLA particles than PLGA particles. With stirring speed of 120 rpm, PLLA particles with average size of 100 to 150 µm were formed. Quite large PLLA particles were prepared without stirring; the average size was between 200 to 300 µm, and the largest particles detected had diameters from 400 to 500 µm. Figure 16 illustrates the size and appearance of the self-prepared particles.

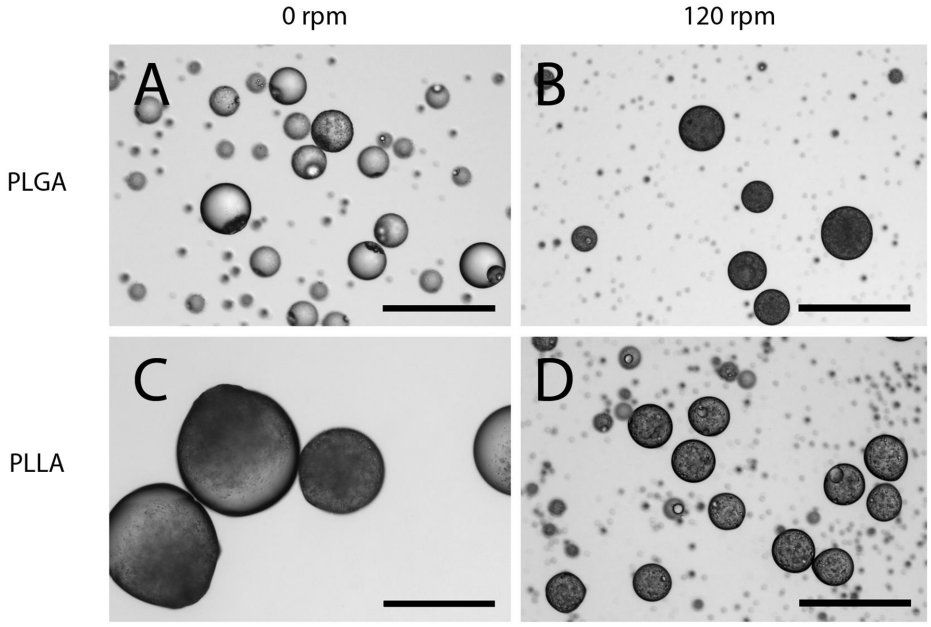

Figure 16. *Size and appearance of self-prepared PLGA and PLLA particles with different stirring speeds. Scale bar 100 µm.*

Without any stirring with a magnetic stirrer stick, smaller size distribution was seen. The beads were rather uniform in size and shape with lower stirring speeds. In contrast, the higher the stirring speed was, the wider was the size distribution and also

more variation in shape of the particles was seen. In addition, with higher stirring speeds the formed particles had more bubbles and granules inside the beads. Furthermore, the particles formed with rougher agitation looked darker in bright field images than particles formed without stirring. An uneven whirl in stirring resulted in particles with irregular shapes; balloon shaped, donut-like and elongated particles were detected (Figure 17). To produce regular spherical particles with single o/w emulsion process seems to require fresh polymer and surfactant solutions.

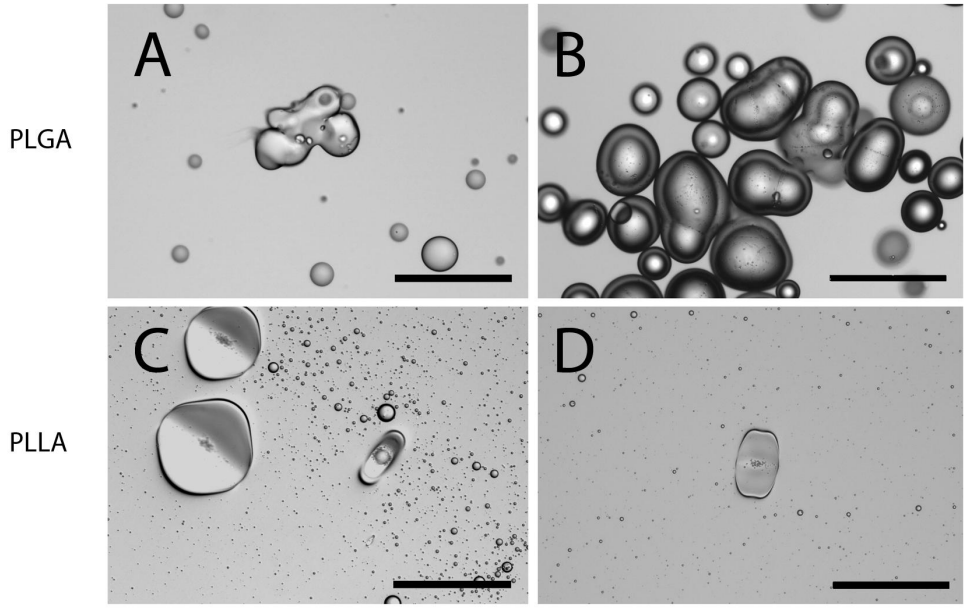

Figure 17. *Irregular structures of self-prepared PLLA and PLGA particles caused by uneven stirring. Scale 200 µm.*

6.1.1. Autofluorescence and background

The self-prepared PLGA particles emitted quite strong autofluorescence with investigated excitation wavelengths of 320 nm, 488 nm and 569 nm. For PLGA particles, the autofluorescence was strongest with the wavelength of 320 nm (UV), but the difference between 320 nm and the other examined wavelengths was not that significant. In addition, also the unprocessed PLGA granules emitted strong autofluorescence with all the investigated wavelengths. The commercial DE 53 particles gave out autofluorescence with all the wavelengths under inspection. Similar to PLGA particles, the autofluorescence for DE 53 was strongest with the wavelength of 320 nm. However, the intensity of the autofluorescence emitted by the DE 53 particles was much weaker than the autofluorescence of the PLGA particles. In contrast to the PLGA and DE 53 particles, the PLLA particles did not show autofluorescence with any of the wavelengths in question.

DAPI caused background with all examined particles. Despite, the background with DAPI was strongest for the PLGA particles. Interestingly, for PLLA and DE 53 the background with DAPI was tolerable and did not prevent imaging with short light

exposure times. The DAPI dilution series was investigated for all the particles. Even with dilutions of 1:1000, DAPI caused significant background for PLGA particles. For PLLA, there was no difference between different DAPI dilutions. The results of autofluorescence and background experiment are listed in Table 10.

Table 10. *Autofluorescence of self-prepared and commercial microparticles and background caused by secondary antibodies.*

Material	*PLGA*	*PLLA*	*DE 53*
Autofluorescence with 320 nm	++++	-	++
Autofluorescence with 488 nm	+++	-	+
Autofluorescence with 568 nm	+++	-	+
Background with DAPI	++++	+	+
Background with DAPI 1:10	+++	-	+
Background with DAPI 1:100	++	-	-
Background with DAPI 1:1000	++	-	-
Background with secondary Abs	Not tested	-	+

6.1.2. Coating of microparticles

Self-prepared PLLA particles and commercial DE 53 microcarriers were coated with mouse laminin and Matrigel™. PLGA was left out of the coating experiment due to its strong autofluorescence feature. Both examined particle types were successfully coated with investigated ECM dilutions. Coating of DE 53 particles with laminin resulted in beautiful uniform coating, which lined the whole surfaces of the particles. The coating was rather thick, and almost all DE 53 beads were fully covered with laminin matrix. Also Matrigel™ coating for DE 53 particles resulted in uniform layer around the particles. However, the laminin coating was thicker compared to the Matrigel™ and resulted in stronger fluorescence when visualised and imaged laminin and matrigel samples simultaneously with same exposure times.

In contrast, for PLLA the coating protocol with both ECM dilutions resulted in only partly coated particles. The coating was not a uniform layer. Instead, areas and spots of coating were seen around the particles. PLLA particles with two different average sizes were studied; 0 rpm (200 to 300 µm) and 120 rpm (100 to 150 µm). For both particle sizes the outcome of the coating was similar. All in all, the success of coating was much better for DE 53 particles, and laminin proved to be slightly better

coating matrix than Matrigel^{TM}. The success of coating procedures for different particle types is illustrated in Figure 18.

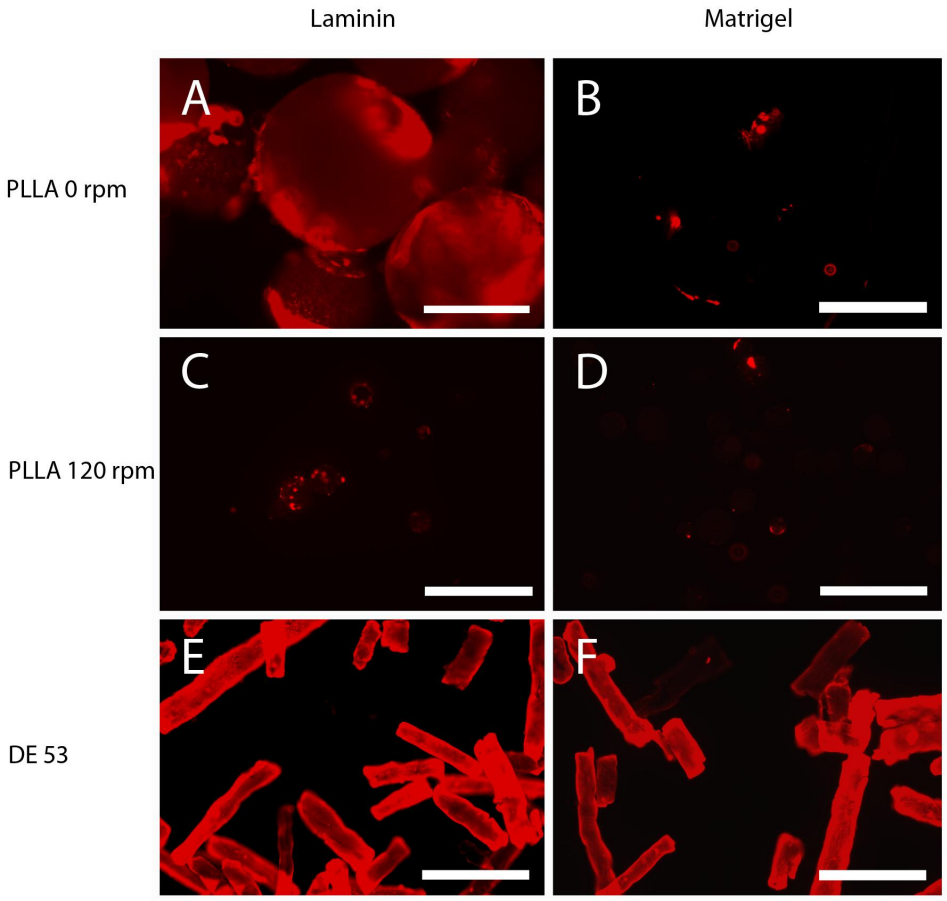

Figure 18. *Immunofluorescence characterization of laminin and Matrigel^{TM} coatings on self-prepared and commercial microparticles using antibody against laminin (red). Scale bar 200 µm.*

6.2. Culturing hPSCs in feeder-free conditions

All examined cell lines, Regea 06/040, Regea 08/023 and FiPS 5-7, were cultured and maintained in feeder-free conditions from 4 to 8 passages (Table 11). The cell lines kept their undifferentiated appearance through their adaption period in feeder-free conditions. Every line under inspection needed time to adapt into feeder-free conditions for at least 3 to 4 passages before they started to proliferate and expand in such amounts that they could been split in ratios of 1:2 and 1:4.

Too small or too large cell colony densities in culture wells led to differentiated cultures and poor cell proliferation. With optimal density, the cell colonies remained undifferentiated and grew rapidly into large and clear lined colonies. If there were areas where the colonies were too close to one another, the colonies differentiated forming follicle like structures. With low cell colony density, the cells did not proliferate in such amounts that they could be split. Time window for passaging hPSCs in feeder-free

conditions was very narrow. In this experiment, the cells were passaged usually 4 or 5 days after the previous splitting, depending on the cell density in wells.

Table 11. *Cell line passages when transferred from feeders into feeder-free cultures and passages adapted in feeder-free before seeding cells on microparticles.*

Cell line	***Passage***	***Passages kept in feeder-free before seeding cells on microparticles***
06/040	p94	5
06/040	p100	4
08/023	p39	5 to 8
5-7	p94	5
5-7	p98	4
5-7	p101	5 to 8

After transferring cells into feeder-free from feeder cells, lots of cell material was lost due to a low attachment of cut cell sheets into Matrigel™ coated wells. Instead, a big portion of transferred cell pieces formed EB-like structures. These unattached pieces were removed 1 to 2 days after transferring hPSCs into feeder free. In addition, in the first 2 to 3 passages in feeder-free, there were quite many differentiated areas, which needed to be removed to keep the cultures as pure undifferentiated hPSCs. Also, few fibroblast-like cells and colonies were seen growing out of stem cell colonies during the first few passages in feeder-free conditions. Differentiated colonies, fibroblast outgrowth and merging colonies are illustrated in Figure 19.

On first day after passaging the cells in feeder-free, the colonies were very small. In addition, the colonies possessed a smooth, clear lined and mainly spherical appearance. The edges of adjacent colonies were far a part from one another. Interestingly, lots of single cells were seen on day 1 after passaging in all cell lines. Especially hiPSC line 5-7 had many of them after every passage. On days 2 to 3 after passaging, the colonies started to grow. They were still clear-lined, smooth and distinct from one another. On day 3, few colonies with denser middles were detected. However, on day 4 more dense centers had appeared. The colonies were also so large that they started to merge with adjacent colonies. By the day 5 after passaging, most of the colonies started to have denser centers with granule-like appearance in light microscope. The appearance of colonies in different time points is represented in Figure 20. Also, the colonies were so large and merged that in some parts they resembled a large uniform cell sheet. At the latest in this point, the cultures were passaged onto fresh Matrigel™ coated well-plates.

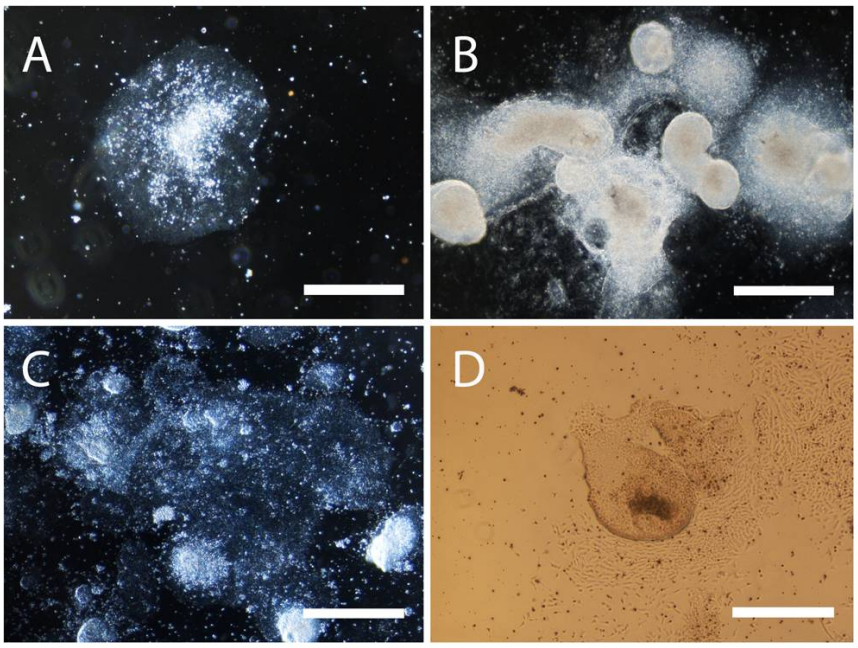

Figure 19. *A. An undifferentiated hESC colony in feeder-free conditions. B. A differentiated hESC colony in feeder-free conditions. C. Merging hiPSC colonies in mTeSR®1. D. Fibroblast outgrowth from a hESC colony in feeder-free conditions. Scale bar 500 µm.*

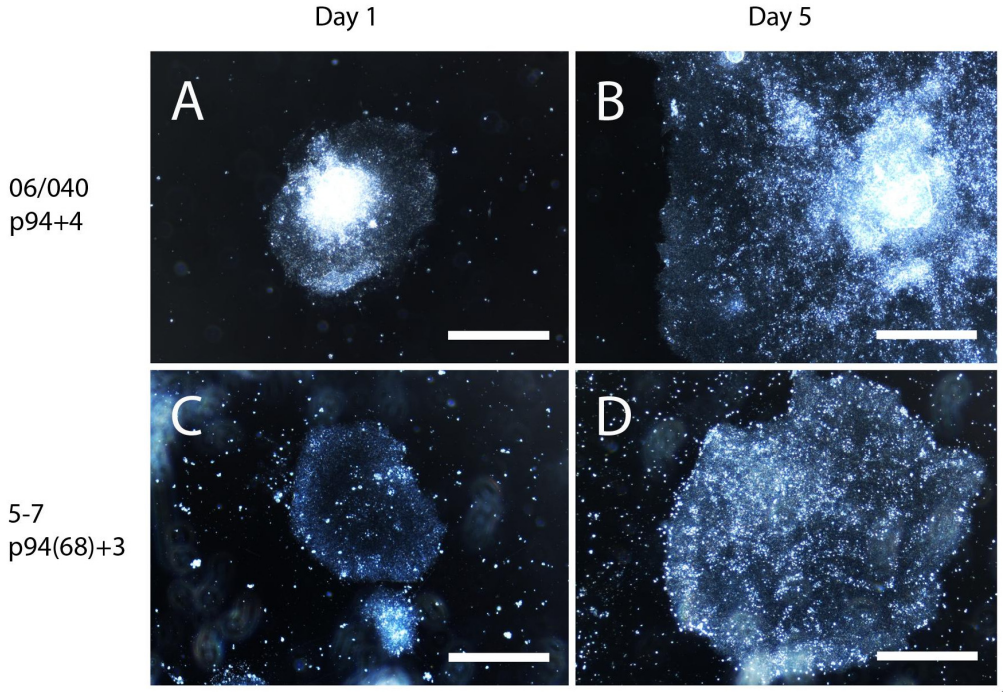

Figure 20. *hESC 06/040 p94+4 and FiPS 5-7 p94+3 in feeder-free conditions. Scale bar 500 µm.*

All the examined cell lines did not prefer the feeder-free environment. Out of the three studied cell lines, Regea 06/040 performed quite poorly when cultured in mTeSR® 1 medium. The cell material was quite impure through out the culture time in feeder-

free conditions even though the differentiated areas were removed regularly. Despite the hESC line 06/040 was successfully maintained in feeder-free conditions up to 5 passages, the cultures did not proliferate in such amounts that it could have been split and increased the cell volume in cultures. On the contrary, FiPS 5-7 and Regea 08/023 lines both grew well in feeder-free conditions. They maintained they undifferentiated nature throughout the culture time in feeder-free, and proliferated in large quantities. FiPS 5-7 and Regea 08/023 also expressed pluripotency markers OCT 3/4 and Nanog after 8 passages in feeder-free environment (Figure 21). However, the intensity of these pluripotency markers when imaged with fluorescence microscope was rather low, and quite high light exposure times were required to be able to visualize these markers.

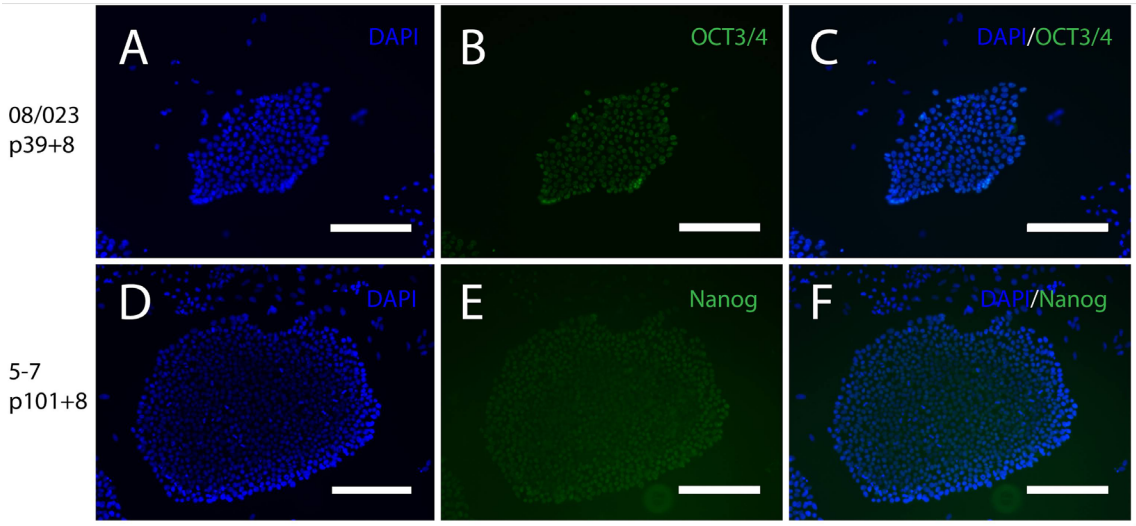

Figure 21. *Pluripotency of hESC and hiPSC lines in feeder-free conditions. Scale bar 200 µm.*

6.3. Attachment and proliferation of hPSCs on microcarriers

Cell lines 06/040, 08/023 and FiPS 5-7 were transferred on to self-prepared Matrigel™ coated PLLA 0 rpm particles with an average particle diameter of 200-300 µm, 120 rpm particles with an average diameter of 100 to 150 µm, and on commercial DE 53 microcarriers. The cells had been adapted into feeder-free conditions from 4 to 8 passages before cell seeding on microparticles. All cell lines attached and proliferated on DE 53 particles depending on the cell to particle ratios. However, the cells attached very poorly to the PLLA beads of both sizes and the cell amount on PLLA particles did not increase. The appearance of microparticles in culture systems is illustrated in Figure 22.

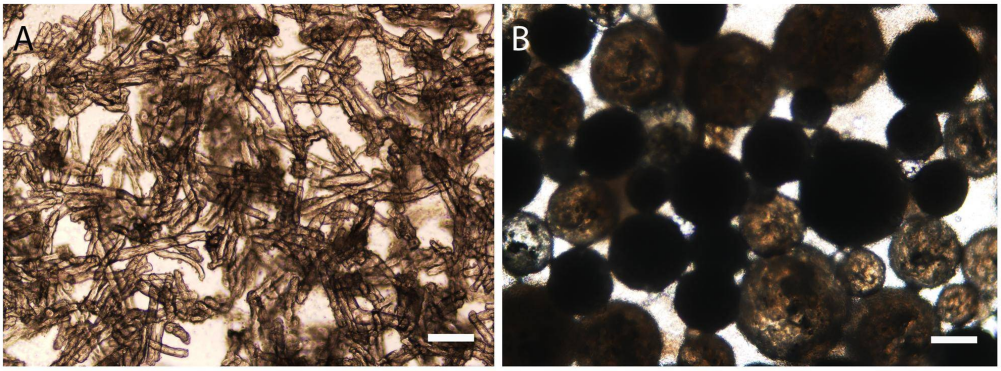

Figure 22. *A. FiPS 5-7 on DE 53 and B. 06/040 on PLLA 0rpm in a bottom of a 96-well plate well. Scale bar 200 µm.*

The cell attachment on particles was analysed with phalloidin and DAPI stainings. DAPI staining showed the attachment of cells on the surface of the microparticles. In all day 3 samples of cells on DE 53, quite many DAPI positive cells were seen. Furthermore, there were phalloidin positive cells in all day 3 samples of hPSCs on DE 53 with beautiful elongated shapes spreading and growing along the particle surface. Interestingly, it seemed that the cells entangled around the beads with their extension. The cells were attached quite evenly on the DE 53 particles, but some clusters of cells were also seen in between the particles. Cell attachment on commercial DE 53 microparticles is represented in Figure 24.

Compared to the DE 53, the cell attachment on the PLLA particles was nearly non-existent. Only few cells had attached on both 0 rpm and 120 rpm PLLA particles. The attached cells on the particles were mainly as small clusters. In addition, there were only couple of phalloidin positive cells visualized on PLLA microcarriers. In addition, an interesting phenomenon was seen in all PLLA samples; the cells had formed EB-like cell clusters (Figure 23). Almost all the cells in these cell clusters were phalloidin positive. In few of these clusters, smaller PLLA particles were trapped inside, but the cells did not seem to grow on them. The cell attachment on PLLA particles in different timepoints is illustrated in Figure 25.

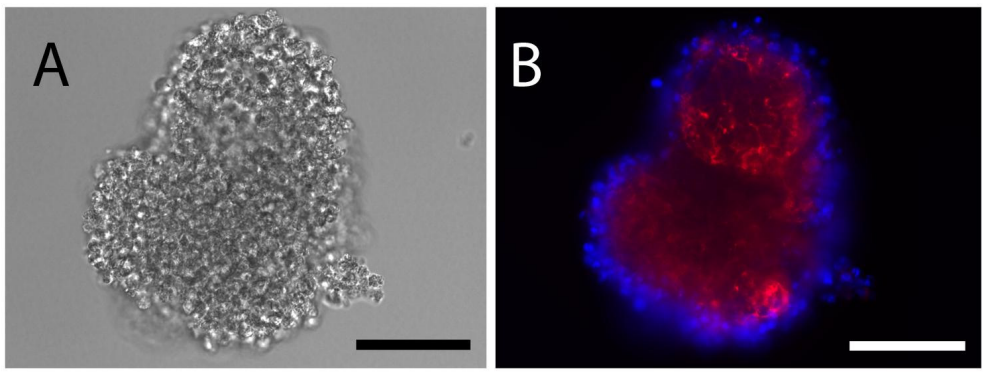

Figure 23. *EB-like cell structures in PLLA cultures. A. A bright field image. B. DAPI (blue) and phalloidin (red) stainings. Scale bar 100µm.*

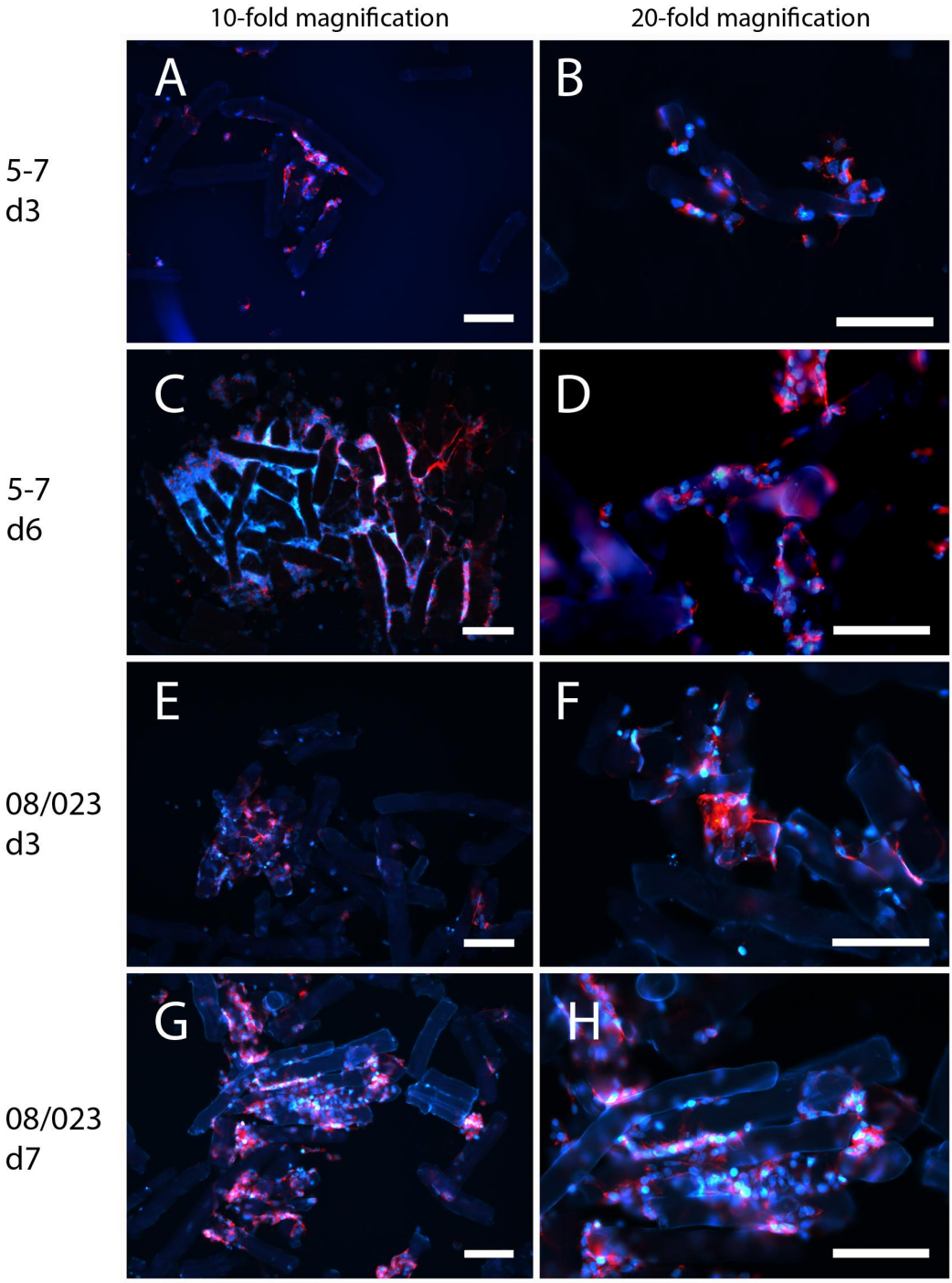

Figure 24. *Cell attachment and proliferation on DE 53 particles. DAPI (blue) and phalloidin (red) stainings. Scale bar 100 µm.*

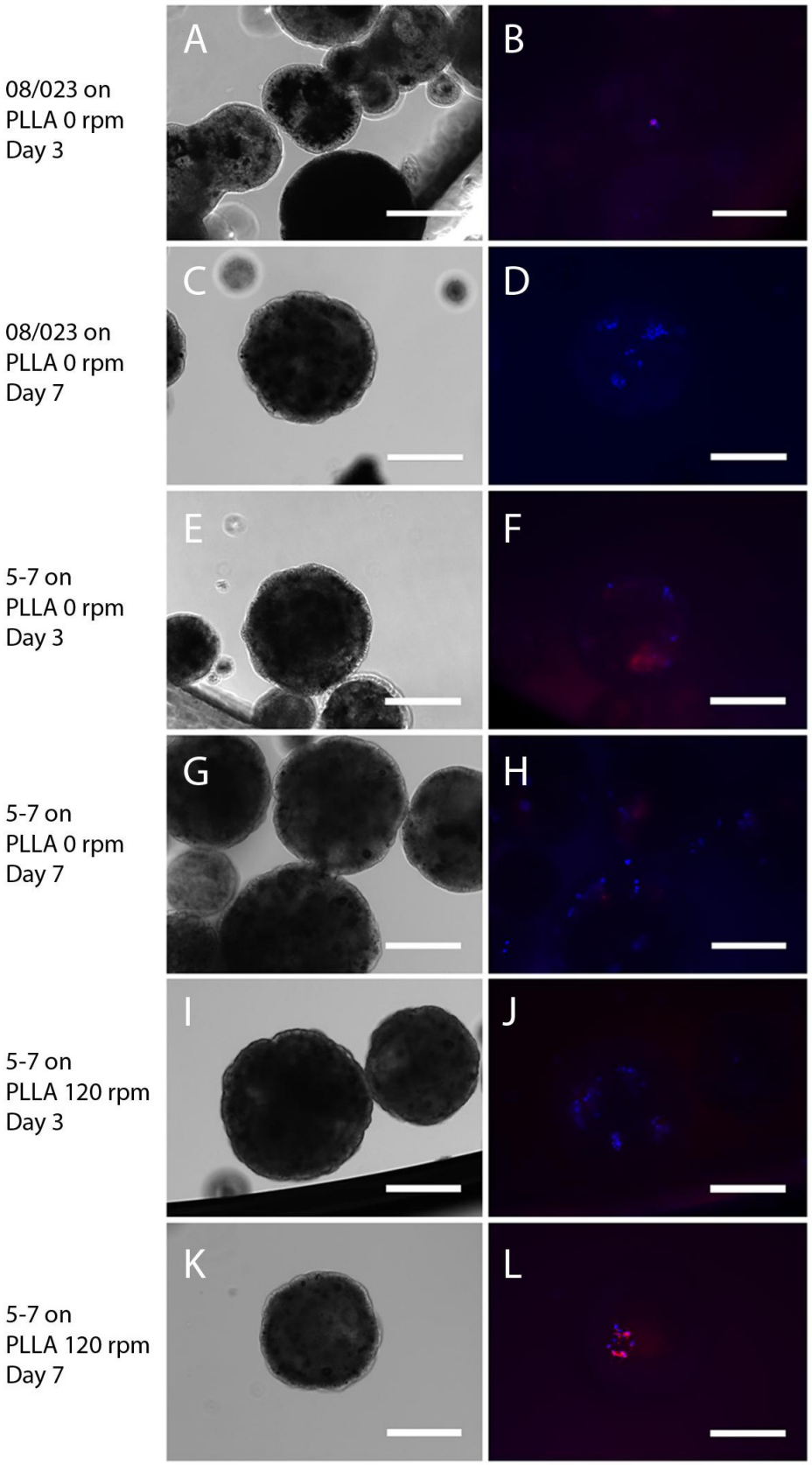

Figure 25. *Cell attachment and proliferation on PLLA 0 rpm and 120 rpm particles. DAPI (blue) and phalloidin (red) stainings. Scale bar 200 µm.*

While inspecting the day 6 and 7 samples of the DE 53 beads, a clear increase in cell amount on particles was seen. Also, the number of phalloidin positive cells had increased. In contrast, there was no increase in cell amount on the PLLA 0 rpm and 120 rpm particles. The amount of proliferating cells on the DE 53 was inspected by Ki67 staining. As result, there were many Ki67 positive cells on these commercial microcarriers. The beautiful phalloidin stuctures on DE 53 particles are shown in Figure 26.

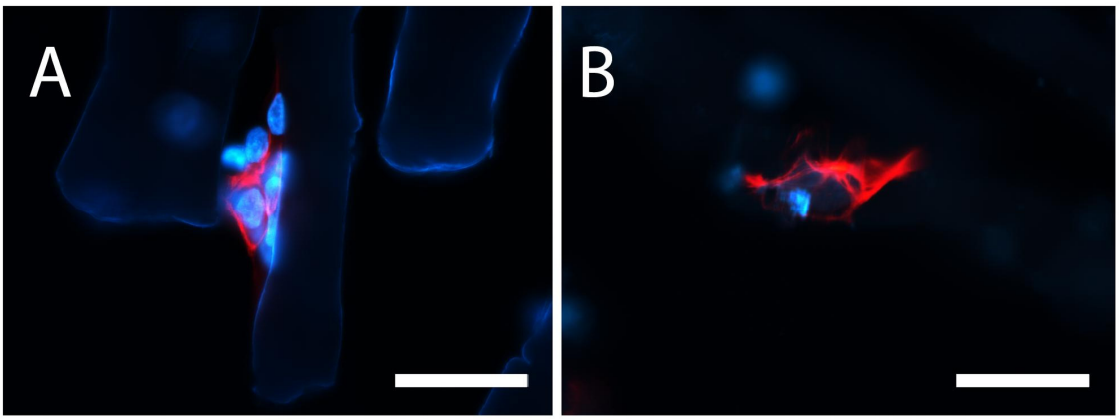

Figure 26. *Cytoskeletons of hiPSCs and hESCs on DE 53. A. 5-7 d7. B. 08/023 d7. DAPI (blue) and phalloidin (red) stainings. Scale bar 50µm.*

The pluripotency of the cells on microparticles was examined with OCT 3/4 and Nanog stainings on day 6 or 7. Visualization of these plurimarkers in the DE 53 and the PLLA samples was extremely challenging. The intensity of the fluorescence of plurimarker positive cells was so low that the background of microparticles almost eclipsed the signals completely. With careful inspection, the OCT 3/4 positive cells were visible for eye with 20-fold magnification in the DE 53 samples (Figure 27), but no Nanog positive cells were detected. However, to be able to take images of these OCT 3/4 positive cells, long light exposure times were required. Yet, with these long light exposure times the autofluorescence and secondary antibody background grew so immense that the plurimarker positive cells were challenging to image. On PLLA particles, no positive cells for the inspected plurimarkers were detected.

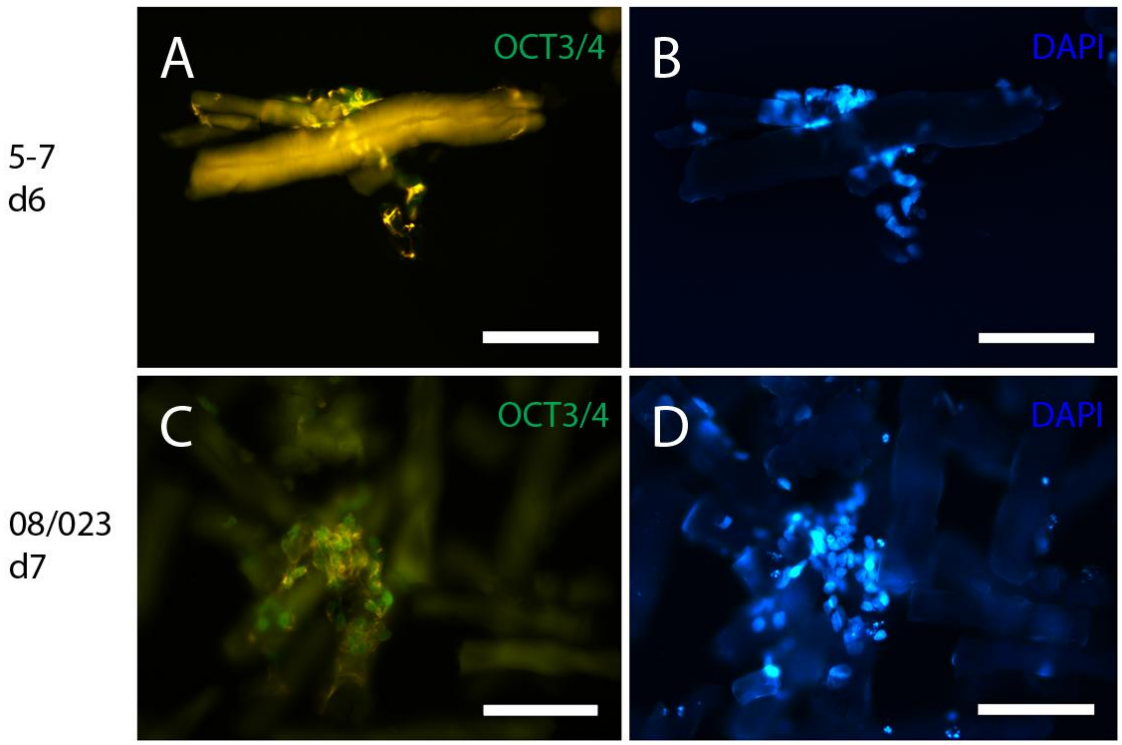

Figure 27. *Pluripotency of hPSCs on DE 53 in feeder-free conditions. A. OCT 3/4 positive and B. DAPI stained FiPS 5-7 cells on DE 53 particles on d6. C. OCT 3/4 positive and D. DAPI stained Regea 08/023 cells on DE 53 particles on d7. Scale bar 100 µm.*

The effect of agitation on cell attachment and proliferation was examined with DE 53 particles in 24-well plate and 96-well plate wells. The agitation had no influence on the amount of attached cells on particles, rather it affected the distribution of the cells on particles. In non-agitated wells, the cells were partly on the other sides of the particles whereas in agitated wells, the cells were quite evenly distributed around the DE 53 microcarriers. Furthermore, in non-agitated wells there were many tight and large aggregates of particles, which did not loosen regardless of vigorous pipetting. The effect of agitation is illustrated in Figure 28. During these experiments with DE 53 particles, it was also detected that more cells attached on particles when smaller well plate size was used. There was much more cells on beads in 96-well plate samples than the ones in 24-well plates with similar cell to particle ratio. Furthermore, in 96-well plate samples there was also much more phalloidin positive cells compared to parallel samples in 24-well plates.

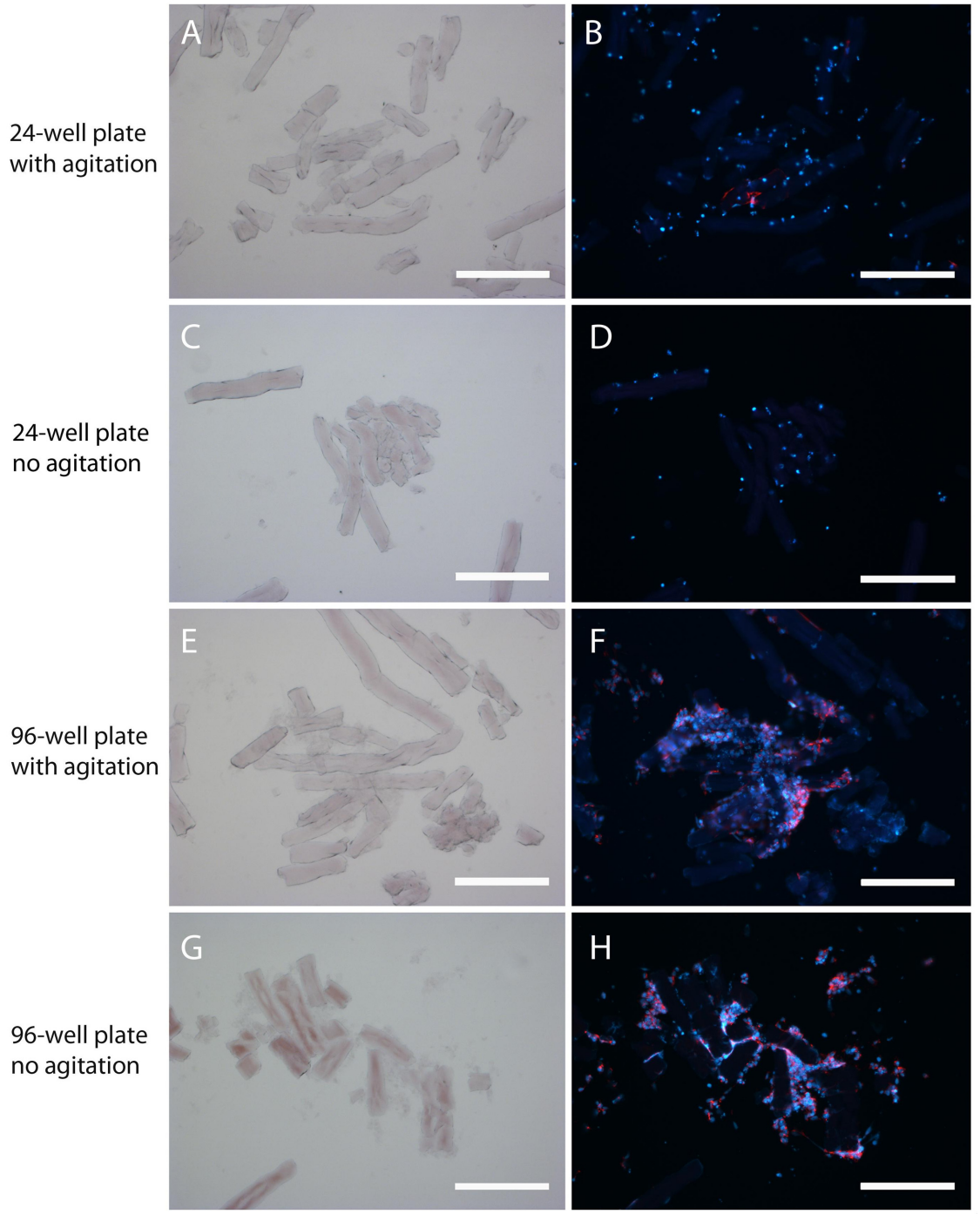

Figure 28. *The effect of agitation and well plate size on cell attachment on DE 53 particles. DAPI (blue) and phalloidin (red) stainings. Scale bar 200 µm.*

The cell to particle ratio seemed to have a huge impact on the number of attached and phalloidin positive cells. The larger the cell to particle ratio was, the more cells had attached on particles and more phalloidin positive cells were detected. The best attachment and proliferation on DE 53 particles was achieved with cell to particle ratios of 100 000 cells/mg and culturing the cells in 96-well plate wells. The results of cell seeding and proliferation on DE 53 particles and PLLA beads are listed in Table 12.

Table 12. *Attachment and proliferation of hPSCs in feeder-free conditions on microcarriers.*

Particle	*Cell line*	*Well-plate*	*Cell to particle ratio (cells/mg)*	*Agitation*	*Cells attached d3*	*Phalloidin positive cells*	*Increase in cell number on d6 or d7*	*Pluripotency*
DE 53	06/040	24	20 000	No	+ +	+	+	OCT 4 positive
DE 53	5-7	96	23 300	No	+	+	+	OCT 4 positive
DE 53	5-7	24	74 000	No	+ +	+	+ +	OCT 4 positive
DE 53	5-7	24	74 000	Yes	+ +	+	+ +	OCT 4 positive
DE 53	5-7	96	100 000	No	+ + +	++	+ + +	OCT 4 positive
DE 53	5-7	96	100 000	Yes	+ + +	++	+ + +	OCT 4 positive
DE 53	5-7	96	100 000	No	+ + +	++	+ + +	Nanog negative
DE 53	08/023	96	100 000	No	+ + + +	+ + +	+ + +	OCT 4 positive
PLLA 120 rpm	06/040	96	9300	No	+	-	-	OCT 4 negative
PLLA 120 rpm	5-7	96	23 300	No	+	+	-	OCT 4 negative
PLLA 120 rpm	5-7	96	100 000	No	+	-	-	Nanog negative
PLLA 0 rpm	5-7	96	100 000	No	+	-	+	Nanog negative
PLLA 0 rpm	08/023	96	100 000	No	+	+	-	Nanog negative

6.4. Differentiation of hPSCs into RPE cells on microcarriers

Pigmented areas were detected in both investigated Regea 08/023 and FiPS 5-7 cell lines 15 days after starting the diffentiation protocol. The pigmented cell clusters were very small, but they were clearly on the surface of the commercial DE53 particles. The amount of pigmented cell clusters increased with the time, and by the day 35 there were approximately 10 to 15 small pigmented areas in both cell lines. During the differentiation time of 5 weeks, the cells formed EB-like structures, where the DE 53 particles were trapped inside the cell clusters. The cell and particle aggregates became so tight that they did not deattach with vigorous pipetting. After 35 days in differentiation, almost all the particles and cells were in EB-like stuctures, and hardly any loose particles were seen.

The differentiation of stem cells into RPE on DE 53 particles was also investigated on gene expression level. The RT-PCR showed that both cell lines express *BEST* and *MITF* genes, which are markers for RPE maturation. In addition, the 08/023 line was positive for *OCT 3/4* plurimarker. Interestingly, the hiPSC 5-7 d0 reference sample for plurimarker also showed *MITF* expression. The –RT controls were all negative. The results of gene expression analysis with RT-PCR are illustrated on Figure 29.

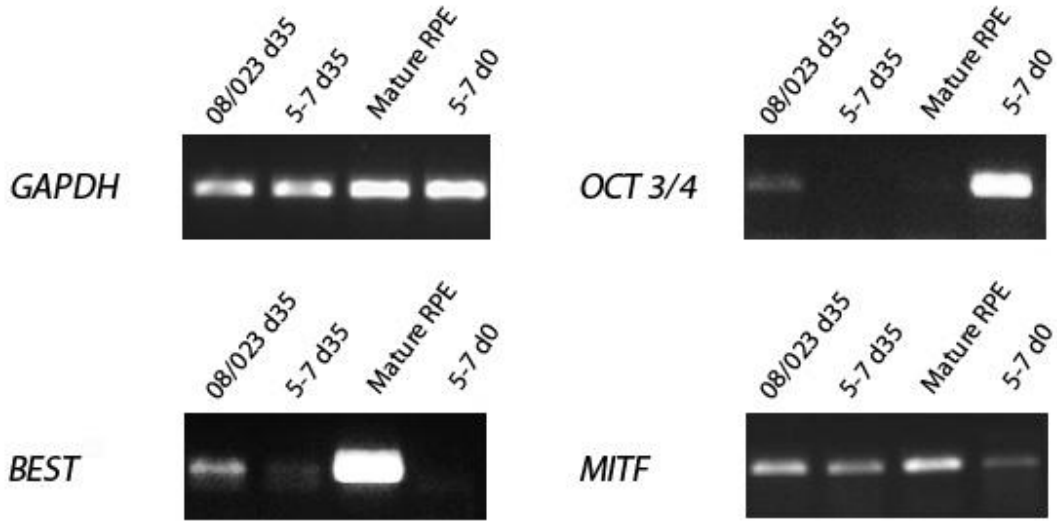

Figure 29. *hPSCs express RPE specific markers on gene expression level after 35 days of differentiation.*

7. DISCUSSION

Degenerative diseases of the retina affect millions of people worldwide. For instance, AMD is the leading cause of blindness in the elderly in the developed countries. (Finnish Federation of the Visually Impaired 2011) Today, these degenerative diseases are incurable, and the current treatments only delay the disease progression. Cell transplantation is considered to be a potential treatment for these retinal degenerations. (Binder et al. 2002) hPSCs are considered to be potential cell sources, especially for the treatment of dry-form of the AMD. However, currently the field of tissue engineering is battling againts sufficient cell production for transplantation and research purposes. The current maintaining methods for hPSCs limit their production in large-scale. Furthermore, the differentiation of these cells into RPE cells is unefficient and results in low yields of mature cells. (Rowland et al. 2011)

In this thesis, the hPSCs were cultured on 3D microparticle cultures in order to improve the efficacy of differentiation of stem cells into RPE cells. In addition, the suitability of microparticle culture systems for maintenance of stem cells in feeder-free conditions was investigated. Self-prepared PLGA and PLLA microparticles were investigated in addition to commercial DE 53 microparticles. The shape and size of the self-prepared particles as well as the cell attachment and proliferation on microparticles were analysed with microscopy and immunofluorescence. RT-PCR analysis was also carried out for inspecting the differentiation of hPSCs into RPE cells in microparticle culture system.

7.1. Particle preparation

The single emulsion process is rather easy, fast and cost effective way to produce large amounts of microparticles with different sizes (Lasselle et al. 2007; Gabler et al. 2007). In this thesis, biodegradable microparticles of PLGA and PLLA were prepared with a single emulsion method according to the protocol of Loo et al. (Loo et al. 2010). Similar emulsion-based protocol has been studied previously with slight modifications for the preparation of PLGA and PLLA microparticles. (Gabler et al. 2007; Bible et al. 2009; Thomson et al. 2010)

The effect of the stirring rate in the resulting particle size in emulsion-based methods has been widely reported. (Lassalle et al. 2007) In this study, the higher the stirring rate, the smaller was the average size of the particles. Furthermore, with higher stirring rates, the size distribution of the particles grew resulting in more heterogenous particles according to their size. The outcome was always a mixed population of different-sized microparticles, never homogenous and uniform. By only changing the

stirring rate and keeping the other parameters constant differently sized microparticles were prepared. The shape and size of the vessels used in particle preparation had an impact on the nature of the whirl in stirring. Most of the studied vessels had a sweeping bottom, which made the stirring uneven. Particles prepared in these vessels ended up to have irregular shapes, such as donut-like and balloon-like. With constant and even stirring, the particles were more uniform in size and shape, which is a strong indication of the importance of stirring in emulsion based particle preparation method. Thus, the stirring affects not only the size of the prepared particles, but also the shape. In this study, the best outcome was achieved with a 100 ml decanter. However, the absence of a permanent and solid lid of the decanters exposed the preparation process for possible impurities and contaminations. A contamination inside a particle would result eventually in a contamination of the cell culture. Another source of impurities is the solvent itself. DCM is toxic for cells, and residuals in particles might lead to extensive cell death in cultures. In this study, the DCM was removed by evaporating overnight. This does not quarantee that all the solvent is removed and the prepared particles might include great amount of solvent residuals. In this study, no analysis was performed to see if there are many residuals left in the particles. In the future, nuclear magnetic resonance spectroscopy could be used for detecting complete DCM removal from the prepared microparticles.

The characteristics of the microparticles prepared by the solvent evaporation technique, especially the size, have been shown to be affected by the concentration of the polymer and polymer blend ratio. (Thomson et al. 2010) The largest particles prepared in this study had diameters between 300 to 400 μm. These large particles were obtained with no stirring. In fact, this is the largest size of particles that could be fabricated with the studied concentrations of PLGA and PLLA. If bigger particles are desired, the concentrations of the polymer solutions need to be increased. In the other studies with PLGA and PLLA microparticles, lower concentrations have resulted in smaller microparticles with more narrow size distribution whereas with higher concentrations larger particles with wider size distributions are obtained. A more narrow size distribution would be useful in order to achieve reproducible and homogenous culture conditions. (Gabler et al. 2007; Thomson et al. 2010) The concentration of the polymer in the solvent used in this experiment was rather low. Nevertheless, the resulting size distribution was quite large, which is in contradiction with the previous results of Thomson et al. and Gabler et al. However, in this study, there was no other investigated concentration as control to which the used concentration and resulting size distribution could have been properly compared to. Therefore, based on this study, nothing can be said about the effect of concentration on the resulting size distribution. The self-prepared PLLA particles were constantly larger than the PLGA particles produced with the similar stirring rates and other parameters. This is most probably due to the more hydrophobic nature and higher i.v. of PLLA. Similar trend have previously been reported for PLLA and PLGA microparticles prepared with single emulsion method. (Gabler et al. 2007; Thomson et al. 2010)

The surface properties of the growth substrata, such as topography and hydrophilicity, influence the cell attachment. In this study, the prepared particles were analysed with light microscopy, which reveals the rough shape and size of the particles, but does not reveal anything about the surface structure and porosity. Commonly, the surface topography of microparticles has been evaluated with scanning electron microscopy (SEM). SEM analysis has shown that increasing concentrations of PLLA in blended particles result in more rough surface structure. (Bible et al. 2009; Thomson et al. 2010) Besides surface topography, the hydrophilicity of the polymer surface is very important for cell attachment. For instance, water-contact-angle technique has been used for evaluating the changes in hydrophilicity of PLGA microparticles. (Bible et al. 2009) In order to evaluate and characterize the self-prepared particles and their suitability for cell culture purposes in more detail, SEM analysis and water-contact-angle studies should be carried out.

Immunofluorescence imaging of biomaterials is often disturbed by autofluorescence of the investigated polymer and other distracting optical phenomena. This is a major disadvantage in analysis of the cells attached on the biomaterial surfaces. The phenomenon can be very intense and it can inhibit the detection of labeled molecules. (Jaafar et al. 2011) Indeed, the autofluorescence was found very disturbing with self-prepared and commercial microparticles also in the experiments of this thesis. Especially PLGA microparticles emitted strong autofluorescence with all the investigated wavelengths, whereas hardly any or tolerable autofluorescence was seen with PLLA and DE 53 particles. The autofluorescence of PLGA could be possible reduced by changing the composition of the polymer. Since PLLA did not emit strong autofluorescence in this study, the increase of lactide/glycolide monomer ratio could result in reduced autofluorescence of the copolymer. Also background with nuclear stain DAPI proved to be disturbing. In order to improve the imaging of the biomaterials, methods to diminish these phenomena need to be applied. For instance, a fluorescence dye with excitation and emission wavelengths outside the spectra of autofluorescence could be chosen (Van de Lest et al. 1995), the polymer scaffold could be sectioned into thin slices (Sung et al. 2004), or alternatively, alkaline phosphatase conjugation of the antibodies could be used to eliminate the need for fluorescence microscopy (Alperin et al. 2005). Unfortunately, most of these techniques are very hard to execute with microparticles and commonly used antibodies. However, a simple material treatment method with a Sudan Black (SB) autofluorescence quenching molecule could provide a solution. The studies have shown that the treatment with SB inhibits the optical phenomena of several biodegradable polymers without interfering with immunofluorescence imaging of the labeled cells cultured on these polymers. (Jaafar et al. 2011)

Several studies have reported improved cell attachment on particles coated with ECM proteins, such as laminin, Matrigel™ and fibronectin. (Bible et al. 2009; Lock et al. 2009; Nie et al. 2009; Oh et al. 2009; Thomson et al. 2010) Despite the popularity of ECM coatings on particles, only few of the studies report confirmation of the presence

of the coating. Bible et al. verified the success of fibronectin coating on PLGA particles by immunofluorescence. (Bible et al. 2009) In this thesis, similar approach was applied to confirm the success of laminin and Matrigel™ coatings on self-prepared PLLA and commercial DE 53 particles. The laminin coating appeared to be slightly better in both particle types compared to Matrigel™. However, the primary antibody used was anti-laminin, whereas Matrigel™ is a mix of ECM proteins of which laminin is only one component. Therefore, the success of Matrigel™ might actually be better than visualized in this experiment. In general, the success of both coatings was rather poor on PLLA particles. There were spots and larger areas of coating, but the majority of the particle surfaces were lacking the protein layer. These results are similar to those reported by Bible et al. for PLGA particles. (Bible et al. 2009) The poor attachment of the coating might be due to the rather hydrophobic nature of the PLLA surface. In contrast, both coatings on commercial DE 53 microparticles were nicely covering the particles. The more successful coating with DE 53 is probably due to the positive surface charge of the particles, which attract the negatively charged ECM proteins. In order to improve the success of coating for PLLA particles, the hydrophilicity or polarity of the PLLA surface should be increased. For example, functionalization of PLGA and PLLA surfaces by plasma polymerization or by surface aminolysis have resulted in improved coating efficacy. (Hong et al. 2005; Bible et al. 2009)

The single emulsion method proved to be a very simple, fast and convenient procedure to prepare biodegradable microparticles in practise. Microparticles with different sizes were prepared by simply changing the stirring rates during the fabrication process. However, more precise and detailed characterization of the prepared particles should be conducted. Furthermore, the surfaces of prepared particles should be modified in order to increase the attachment of ECM proteins. A major limitation of the studied materials, especially PLGA, is the strong autofluorescence of the polymers, which disturb the analysis of cells grown on the surfaces of the particles. Thus, methods to decrease these disturbing phenomena should be investigated.

7.2. Culturing hPSCs in feeder-free conditions

Currently, most of the stem cell lines are maintained on feeder cells. However, maintaining stem cells on feeders is both laborious and expensive. Furthermore, the effect of feeder cells on stem cells is not yet completely unravelled. (Hakala et al. 2009) In this study, we followed the protocol of Oh et al. who cultured hESCs on commercial DE 53 particles in feeder-free conditions. (Oh et al. 2009) However, transition of cells straight from feeders and from 2D structure into feeder-free 3D cultures with different media could be fatal for the sensitive hPSCs. Therefore, the hPSCs were adapted in feeder-free conditions in 2D cultures for several passages before seeding cells on self-prepared PLLA and commercial DE 53 particles. Despite laminin proved to be a slightly better coating material compared to the Matrigel™, Matrigel™ was used with the feeder-fee culture medium mTeSR®1. The mTeSR®1 feeder-free culture system has

been optimized for Matrigel™ and changing this matrix to another would have required optimization of the culture conditions. Furthermore, Matrigel™ has been shown to be essential for stable long-term propagation of hESCs in microparticles (Chen et al. 2011).

The investigated feeder-free method supported the growth of undifferentiated hPSCs. All the investigated cell lines were maintained successfully in mTeSR®1 media and on Matrigel™ coated wells for 4 to 8 passages. However, during the transmission of the cells into feeder-free, lots of cell material was lost. In some cases, even half of the cut pieces did not attach to the new growth substrata, instead they formed EB-like structures. This poor attachment of cell pieces could result from cutting the pieces too large, which might hinder the attachment of cells. In addition, the lack of growth factor secreting feeder cells and transfer into probably less abundant medium might be too shocking for some of the cells. Moreover, the cells were seeded on Matrigel™, which suffers from batch-to-batch variation due to its origin in mouse Engelbreth Holm-Swarm sarcoma cells.

In all cell lines, a lag phase in the growth and proliferation was seen after transferring the cells into feeder-free cultures. This lag phase usually took 3 to 4 passages during which the cell amount hardly increased. After the initial lag phase, the cells proliferated nicely. Similar lag phase with stem cultures in mTeSR®1 medium has been previously reported. (Ojala 2009) The lag phase might result from too small cell seeding density in wells when transferring cells to feeder-free conditions. Furthermore, the cells might need time to adjust to the new culture conditions. However, this adjustment might be dangerous if it involves changes in gene expression and chromosomal level. hESC lines derived and cultured in feeder-free cultures have shown genetic instability and chromosomal abnormalities. These abnormalities in karyotypes are not welcome when maintaining stem cell lines. (Ludwig et al. 2006a; Catalina et al. 2008) In this thesis, the stem cells were cultured in feeder-free conditions for maximum 8 passages. This is a rather short time scale to evaluate the success of a culture method, and no conclusion of long-term support of undifferentiated stem cells in this feeder-free culture system can be drawn based on this study. However, long-term cultivation of stem cells in mTeSR® 1 has been shown to support the undifferentiated state of hESCs with no chromosomal changes and abnormalities. (Ojala 2009)

The studied feeder-free culture method in this thesis was very sensitive. Especially during the first few passages, there were quite many differentiated areas, which needed to be removed in order to maintain the culture pure. The low quality of the cultures in the beginning might be due to inappropriate cell seeding density. Thus, in the beginning of feeder-free cultures, a great number of cells are required to start up a pure and healthy culture. Also, during the time of the experimental works, the stem cell lines under investigation were performing quite poorly. Hence, it is understandable that the resulting feeder-free cultures suffered and struggled in the beginning. The time window for passaging hPSCs was very narrow. Therefore, the cultures needed close inspection daily. The cell density and distribution of colonies also had an affect on the purity and pluripotency of the colonies. Too sparse or dense cultures resulted in

differentiated areas. The outgrowth of fibroblast-like cells was detected in all cell lines during the first few passages of stem cells in 2D feeder-free cultures. These fibroblast-like cells have been also previously detected in hESC colonies cultured in feeder-free conditions. (Stojkovic et al. 2005; Wang et al. 2005; Ojala 2009)

Out of the three stem cell lines studies, hESC line Regea 08/023 and hiPSC line FiPS 5-7 proliferated nicely after the lag phase in the beginning. Unfortunately, the hESC 06/040 line survived rather poorly in feeder-free cultures, and these cells were only maintained, not proliferated in feeder-free conditions. The poor success of 06/040 cell line can result from the bad outperformance of the line in general during the time of the experimental work. Some hESC lines have also been reported to have poor survival when passaged enzymatically (Li et al. 2009). Addition of ROCK inhibitor to the culture media might increase the cloning efficiency of hESCs in enzymatically passaged cultures. The Regea 08/023 and FiPS 5-7 feeder-free cultures were pure and the colonies clear-lined and spherical. There were much less differentiated areas detected in the later passages. Both of these lines expressed pluripotency markers after 8 passages in feeder-free conditions. Despite, more detailed and thorough analysis on gene expression level should be conducted in order to evaluate the pluripotency of the investigated lines than conducted in this experiment. Moreover, more detailed investigations are required before conclusions can be drawn about the suitability of specific lines for feeder-free conditions.

Feeder-free culture system with mTeSR®1 medium and Matrigel™ offers a culture solution which takes the culture of stem cells towards xeno-free and defined culture systems. Even though the mTeSR®1 the feeder-free culture system is fully defined, it includes animal protein sources in the culture media and it is combined with animal-based Matrigel™, which in fact, makes it a non xeno-free method. (Ludwig et al. 2006b) However, new human-derived counterparts for Matrigel™ have been developed and are commercially available. The use of these human-based ECM components would remove one animal-based component from the feeder-free culture systems. After the lag phase in the beginning of feeder-free cultures, the maintenance of these cells was rather convenient. In a feeder-free culture system, there is no cultivation and maintenance of the feeder cell population, and the enzymatic passaging of these cells in the feeder-free conditions was relatively fast and easy compared to the conventional mechanical passaging of stem cells on feeders. An effective feeder-free culture method without any animal-based components could provide a more convenient and cost effective culture system alternative for conventional stem cell cultures on feeder cells. However, more detailed and profound comparison of the effectiveness and costs between conventional culture system on feeders and feeder-free culture systems should be carried out.

7.3. Attachment and proliferation of hPSCs on microparticles

The attachment and proliferation of hESCs on microparticles have been studied on several different particle types (Crook et al. 2008; Fernandes et al. 2009; Leung et al. 2009; Lock et al. 2009; Nie et al. 2009; Oh et al. 2009; Storm et al. 2010; Chen et al. 2011). In this thesis, the attachment and proliferation of Regea 08/023 and FiPS 5-7 lines were investigated on self-prepared PLLA particles of two sizes and commercial DE 53 microparticles in feeder-free culture conditions. The self-prepared PLGA particles were not chosen for the cell culture studies due to their immense autofluorescence and background. The effect of different cell to particle ratios, agitation, and well-plate sizes in cell attachment to these particles were tested. The attachment and proliferation of investigated stem cell lines were evaluated by immunofluorescence stainings.

The cells attached poorly on the self-prepared PLLA particles of both sizes. Furthermore, the cells did not proliferate during the 7-day culture time on microparticles. There was no difference in cell attachment and proliferation between the smaller and larger PLLA particles. However, the smallest particles were rarely seen when analysing the results. They were probably lost during the immunofluorescence protocol due to their small size and slow sedimentation to the bottom of the tube or they were trapped inside the EB-like cell clusters. The smaller spherical particles have larger surface area to volume ratio. Therefore, similar cell to particle ratios used in sell ceeding should result in higher yields with the smaller particles due to the increased surface area. However, too small particles size has been shown to result in aggregate formation (Chen et al. 2011). To investigate the effects of size more thoroughly, more controlled shapes and size distributions of microparticles are needed.

In this study, the few cells visualized on the investigated PLLA particles were attached as spot-like small clusters or as single cells on microparticles. In fact, the cell attachment on these biodegradable particles was quite similar to the attachment of the ECM coatings. It is possible that the cells were able to attach only to the coated areas on particle surfaces. Therefore, poor coating success might have resulted in low cell attachment and proliferation. However, the overlapping of the coating and cells should be verified by immunostaining.

Based on this study, hPSCs do not prefer PLLA microparticles as a culture substrate. This might be due to the rather hydrophobic nature of the particle surfaces. Furthermore, there might have been solvent residuals left in the prepared particles, which might have been toxic for the cells. However, PLGA microparticles, which were prepared with similar preparation protocol and residual removal as done in this study, supported the attachment of neural stem cells (Bible et al. 2009). Nevertheless, more thorough purification of the particles after preparation is in order to ensure the purity of the particles. For example, Thomson et al. incubated blended PLLA and PLGA particles prepared with similar single emulsion method overnight in 70 % ethanol to remove the

solvent residuals (Thomson et al. 2010). In addition to solvent residuals, the acidic degradation products of PLLA might have disturbed the growth of the sensitive hESCs. However, the culture time in this experiment was rather short during which the degradation of PLLA should not be meaningful.

In contrast to the PLLA microparticles, hPSCs attached and proliferated well on the commercial DE 53 microparticles. Cellulose is quite hydrophilic material due to the many hydroxyl groups in its molecular structure. The hydrophilic nature of DE 53 microparticles in combination with positively charged surface might have increased the cell attachment. These results are in line with the results of other research groups investigating maintenance of hESCs on DE 53 particles in feeder-free conditions. (Oh et al. 2009; Leung et al. 2010) However, the findings in this study were based on immunofluoresrence stainings, and therefore, they are not quantitative. The analysis of cell proliferation should be verified for example by cell counts or by comparing the amounts of RNA between two timepoints. Commercial cell proliferation assay kits provide an alternative method for measuring cell proliferation, as well. The DE 53 microparticle culture system seemed to be suitable for maintaining hPSCs in undifferentiated state. Most of the cells expressed a plurimarker after 6 to 7 days in microparticle cultures. However, the visualization of the plurimarker proved to be extremely challenging due to the strong background and autofluorescence of the particles. Therefore, other methods for the analysis of the pluripotency should be applied. For instance, fluorescence activated cell sorting (FACS) and RT-PCR have been used for analysing the pluripotency of hESCs on DE 53 particles. (Oh et al. 2009) Furthermore, in this experiment, only few plurimarkers were examined. Solely feeder-free conditions have shown to be prone for genetic alterations and abnormalities for hESCs (Ludwig et al. 2006a). This in combination with a 3D growth substratum with strong positive charge can result in an environment which exposes the cultures for genetic alterations or induces differentiation of hPSCs. Therefore, the cells cultured in these conditions should be karyotyped and their teratoma formation capability should also be checked in the future.

The cell to particle ratio had a huge impact on the cell attachment and proliferation. The more cells were seeded relative to microparticles, the more cells attached and proliferated. In cultures with too sparse cell density, the cells lack essential cell-to-cell interactions and the amount of secreted growth factors is minor, which might have led to worse cell proliferation in cultures with smaller cell to particle ratio. Therefore, initial parameters for microparticle cultures have significant influence on the cell attachment and proliferation efficacy and require optimization in future studies. Addition of ROCK inhibitor to the culture medium might have improved the cell attachment and proliferation. Increased cell survival of hESCs in microparticle cultures with ROCK has been reported (Storm et al. 2010).

The major advantage of the microparticles is their large surface area compared to the 2D cultures. In this study, the absence of continuos agitation allowed the particles to settle down to the bottom the well. This decreased the available surface area for cell

attachment. The particles also settled quite tightly, which might have led to the formation of concentration gradients and inefficient gas-liquid oxygen transfer. Thus, the absence of continuos agitation decreases the factors considered to be advantages of microparticle culture systems. In order to fully utilize the potential of microparticle culture system, continuos agitation is most likely required. In this study, the effect of agitation during cell seeding on cell attachment was investigated. The agitation did not have an influence on the amount of attached cells. Instead, in cultures with agitation, the cells were more evenly distributed on the particles. In addition, there was less and smaller aggregates in cultures with agitation. These results are similar to previously reported by Storm and his colleagues for mESCs in microparticle cultures. (Storm et al. 2010) Agitation in microparticle cultures causes fluid flow in the culture flasks. In large-scale cultures, such as in spinner flasks and bioreactors, the forces caused by the continuos agitation are significant and might induce signalling responses in the cultured cells. For example, the agitation has been shown to induce differentiation of hESCs in microparticle cultures (Leung et al. 2010). Thus, the effects of agitation should be investigated more thoroughly, and a broader comparison between cell lines should be conducted.

Different parameters of microparticles and microparticle culture conditions have not yet been thoroughly studied. Among these is the shape of the microparticles. In this study, microparticles of two different shapes, cylindrical and spherical, were tested, but the influence of the shape on cell attachment and proliferation was not evaluated. However, it was noticed that the cells gathered between DE 53 particles that had adhered together. The elongated shape of the cylindrical DE 53 microparticles might promote adhesion of microparticles, because these particles have more surface area where the adhesion can take place compared to spherical particles. Similar aggregate formation with cylindrical particles in hESC cultures has been reported (Chen et al. 2011). The importance of microparticle shape might be emphasized in agitated large-scale cultures. The shape of the particle might influence on the shear stress that the cells experience, because cylindrical microparticles are anisotropic. The cells on different sides of the cylindrical microparticles in agitated cultures might experience the shear stress with different forces resulting in more heterogenous culture conditions, whereas in spherical ones the shear stress affecting the cells would presumably be more homogenous.

In this thesis, the hPSCs were cultured on microparticles for a very short period. After 6 to 7 days in microparticle cultures, the DE 53 particles had a great number of cells growing on them, but the particles were not fully confluent yet. Furthermore, the yields of proliferating stem cells on microparticles were not properly compared to the 2D cultures. Thus, nothing can be concluded about the efficacy and long-term effects of microparticle culture system compared to the conventional culture systems based on this study. Despite, the success of culturing hESCs on DE 53 particles in feeder-free conditions has been shown also in long-term studies (Oh et al. 2009). In addition, the culture systems in this experiment were very small, whereas the most promising results

of maintaining hESCs on microparticles have been detected in large-scale cultures, such as spinner flasks and bioreactors. (Oh et al. 2009; Storm et al. 2010; Fernandes et al. 2009) In these studies, 2 to 4 fold yields compared to the conventional 2D cultures have been reached in short periods. Thus, the microparticle culture systems might be more efficient in large-scale culture systems.

The cell attachment, distribution and proliferation in this study were evaluated by immunofluorescence staining. Besides the autofluorescence and background with DAPI, secondary antibodies and phalloidin also disturbed the analysis of cells on microparticles. Furthermore, the microparticles are 3D structures, with cells growing around the particles. In immunofluorescence imaging, these 3D structures were attempted to be converted into 2D images. Obviously, plenty of information was lost while imaging of these 3D stuctures by conventional fluorescence microscopy, and most of the visualized cells were out of focus. In addition, the 3D structure of investigated microparticles became a problem when plating them between objective and coverage glass for the visualization of the immunofluorescence analysis. Especially the PLLA particles were so thick that huge amount of mounting media with DAPI was required. This resulted in increased background during the imaging. Therefore, more practical imaging methods appropriate for 3D structures, such as confocal microscopy, should be investigated and further developed.

Based on this thesis, and similar results from the literature, the commercial DE 53 microparticles seem suitable for maintaining and proliferating hPSCs in feeder-free conditions, whereas the self-prepared PLLA microparticles do not support the attachment and proliferation of hPSCs. However, more thorough characterization and long-term culture of the cells in microparticle suspension cultures should be conducted. Furthermore, building and designing a large-scale microparticle culture system with continuos agitation could result in huge yields of undifferentiated hPSCs. This system could provide a potential and cost-effective cell source for research purposes and tissue engineering applications. Despite the suitability of DE 53 microparticles for *in vitro* applications, they are not potential candidates for transplantation purposes due to their non-biodegradable nature. In contrast, the PLLA microparticles offer the advantage of being biodegradable and allow incorporation of therapeutic agents or growth factors inside and can function as PAMs. These characteristics would be beneficial both *in vitro* and *in vivo* tissue engineering applications. Therefore, an effort to increase the cell attachment and proliferation of hPSCs on these PLLA microparticles should be carried out.

7.4. Differentiation of hPSCs into RPEs on microparticles

The current differentiation protocols for RPE differentiation from hPSCs are inefficient and result in very small yields of mature RPE. Moreover, in these protocols, the differentiation is spontaneous, not directed. (Klimayanska et al. 2004; Lund et al. 2006; Vugler et al. 2008; Idelson et al. 2009; Meyer et al. 2009) Therefore, there is a demand

for more effective differentiation protocols. Microparticles have been utilised in maintenance and proliferation of hESCs in suspension cultures, but only few studies have reported differention of hESCs towards mature cell types. (Phillips et al. 2008; Lock et al. 2009; Lecina et al. 2010; Leung et al. 2010) In this study, the capability and efficiency of stem cells to differentiate towards RPE cells on microparticles was investigated. The differentiation of hPSCs into RPE cells in microparticle culture system has not been previously reported in the literature.

Due to the poor cell attachment on PLLA particles, solely the suitability of commercial DE53 particles for differentiation of Regea 08/023 and FiPS 5-7 into RPE cells was tested. The hESC line 06/040 was left out from differentiation studies due to its poor performance in feeder-free culture conditions and cell attachment experiments. The investigated cells were adapted in feeder-free cultures for 8 passages after which they were seeded onto Matrigel™ coated microparticles. After two days of culturing microparticles in mTeSR® 1 media, the RPE differentiation media DM- was changed to the cells.

The appearance of pigmentation is considered to be a sign of RPE differentiation and maturation. (Burke et al. 2008; Gamm et al. 2008) The first pigmented areas in both investigated cell lines were detected on day 15 after starting the differentiation protocol. The pigmented cell clusters were clearly on the surfaces of the particles. The appearance of pigmentation was quite similar compared to the conventional differentiation protocol in use in our group. Thus, the differentiation of hPSCs into RPE cells is not accelerated by microparticle culture conditions compared to the conventional EB culture method (Vaajasaari et al. 2011). During the differentiation, the number of pigmented areas on both lines increased. However, the pigmented cell clusters were small. After 35 days in differentiation media, 10 to 15 small pigmented areas were seen. The amount of pigmented cells was not larger compared to the conventional differentiation protocol (Vaajasaari et al. 2011). These conclusions are based only on visual estimation of the amount of pigment in the cultures, which is objective and not quantitative data.

During the differentiation, the particles started to aggregate, and cell bridges started to grow between the small aggregates. After five weeks in differentiation, these aggregates had formed EB-like structures, which did not detach. The aggregation and formation of these EB-like structures might have had influence on the differentiation. Therefore a constant agitation of the particles in long-term cultures would be beneficial for preventing the clustering of particles. The formation of EB-like structures in hESC microparticle culture systems has been reported previously with high cell seeding ratios. (Lock et al. 2009) Indeed, the cell to particle ratio in the differentiation experiment was rather high. The seeded cells probably proliferated in such amounts, that the beads became confluent, and there was no substrata for all the cells where attach to. In a recent study, the commercial DE53 particles became confluent of hESCs in mTeSR® 1 feeder-free culture conditions on day 6. (Oh et al. 2009) The cell to particle ratio used in the study in question was 1.5-fold compared to seeding ratio used in this experiment.

Besides a constant agitation, passaging of cells onto fresh particles might decrease the formation of EB-like structures.

In addition to observing the appearance of pigmentation, the differentiation was investigated on gene expression level. The RT-PCR analysis showed that both investigated cell lines expressed RPE maturation markers bestrophin and MITF. Thus, both hESCs and hiPSCs differentiate into RPE cells on commercial DE 53 microparticles. The bands visualised in gel electrophoresis were quite weak, especially for the bestrophin, but they were still visible. Interestingly, the pluripotent control sample of undifferentiated hiPSCs also expressed MITF. This might be due to the hFFs on which the control sample was grown. The expression of MITF in human fibroblast cells has been previously reported (Semov et al. 2002). The RT-PCR is a qualitative analysis method, which reveals which genes are expressed in the investigated samples. However, it does not reveal anything about the expression levels of the genes. Therefore, in order to evaluate the effectiveness of the differentiation in microparticle culture system, quantitative analysis such as quantitative RT-PCR should be carried out.

Based on this experiment, hPSCs can differentiate to RPE cells in microparticle suspension cultures. However, it seems that the differentiation efficacy is not increased when compared to the conventional culture method during the first five weeks. (Vaajasaari et al. 2011) More thourough investigations are required before final conclusions can be drawn regarding the differentiation efficacy. As the differentiation on conventional EB-cultures, the differentiation on microparticles seems to be spontaneous. The development and maturation of RPE is a rather slow process, both *in vivo* and *in vitro*. (Klimayanska et al. 2004; Vugler et al. 2007) Thus, a long-term effect of differentiation of stem cells towards RPE in microparticle culture systems on the resulting yields should be investigated. Even though the microparticles seem not to have an influence on the differentiation in short-term, they might result in increased yields in long-term studies.

8. CONCLUSIONS

The traditional culture methods of hPSCs prevent their production in large-scale and are expensive and laborious. Therefore, novel culture methods are constantly being searched. Furthermore, the current differentiation protocols for RPE cells from hESCs are inefficient resulting in low amounts of cells available for research purposes. The aim of this thesis was to investigate if self-prepared biodegradable PLGA and PLLA and commercial DE 53 microparticles could be utilised in increasing the efficacy of RPE differentiation from hPSCs. In addition, the suitability of microparticles for hPSC maintenance in feeder-free culture conditions was investigated. No previously published data exists about RPE differentiation in microparticle suspension cultures from stem cells.

Self-prepared PLLA microparticles did not support the attachment and proliferation of hPSCs in feeder free conditions. The PLGA microparticles were not included in cell culture studies due to their enormous autofluorescence characteristic. The poor performance of PLLA particles in cell culture studies is probably due to the hydrophobicity of the particle surfaces, which hinders the attachment of ECM coatings and cells. However, these PLLA particles are biodegradable and offer the advantage of incorporation and controlled release of pharmacologically active agents, which makes these particles attractive for tissue engineering applications. Thus, effort should be made in order to increase the cell attachment on these particles.

In contrast to PLLA microparticles, the commercial DE 53 particles supported the growth of hPSCs in feeder-free culture conditions in short-term studies. Furthermore, the hPSCs were successfully differentiated into RPE cells on these DE 53 particles. However, the differentiation efficacy in these microparticle suspension cultures was not increased compared to the conventional differentiation protocols during the five-week experiment time. Nevertheless, the long-term outcome of the differentiation on microparticles should be investigated in the future.

The analysis of cell attachment and proliferation on microparticles is complicated by the immense autofluorescence of the particles as well as background with secondary antibodies and DAPI. Furthermore, the 3D structure of the particles prevents their proper analysis with conventional immunofluorescence protocols and microscopy. Therefore, novel methods for characterization and imaging of cells on microparticles should be developed.

Microparticles are far from being routinely used in hPSC maintenance and proliferation. However, at the moment they seem appealing and promising with possibilities to grow stem cells in large-scale and cost effectively. This thesis confirmed

the previously reported suitability of commercial DE 53 microparticles for the maintenance and proliferation of hPSCs. Moreover, this was the first study in which hPSCs were successfully differentiated into RPE cells on microparticles. However, the effectiveness of the differentiation in microparticle suspension cultures did not rise above the conventional differentiation protocols, at least in based on these preliminary short-term studies.

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